DEVELOPMENT AND VALIDATION OF A LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS ANALYSIS OF AMLODIPINE, VALSARTAN AND HYDROCHLOROTHIAZIDE

A thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Pharmacy in Pharmaceutical Analysis of the University of Nairobi

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Declaration

This research thesis is my original work and has not been presented elsewhere for research work.

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DEDICATION

This thesis is dedicated to my family members who were very patient with me during the time I was doing this project and for their financial and moral support. I could not have made it without their motivation and encouragement.

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ABBREVIATIONS

ACE	Angiotensin converting enzyme
ACN	Acetonitrile
AMLO	Amlodipine
API	Active pharmaceutical ingredients
ATI	Angiotensin II type 1
ATR-IR	Attenuated total reflection-IR
BP	Blood pressure
CRS	Chemical reference standard
CV	Coefficient of variance
°C	Centigrade (degrees)
HCTZ	Hydrochlorothiazide
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
IR	Infrared
ICH	International Committee on Harmonization
k'	Capacity factor
LOD	Limit of detection
LOQ	Limit of quantitation
M	Molar (concentration)
mg	Milligrams
Min	Minutes
ml	Milliliter
mm	Millimeter
RSD	Relative standard deviation
R^2	Coefficient of variation
S/N	Signal to noise ratio
SN	Serial number
t_R	Retention time
µ	Micron
µL	Microliter
µm	Micrometer
UK	United kingdom
UPLC	Ultra performance liquid chromatography
USP	United States Pharmacopoeia
UV	Ultraviolet.
UV/VIS	Ultraviolet/Visible
USA	United States of America
VAL	Valsartan
λ_{max}	Wavelength of maximum UV absorption

ABSTRACT

Treatment of hypertension using one drug is desirable because compliance is likely to be better, lower overall cost and fewer adverse effects. However, most patients with hypertension require two or more drugs, preferably acting by different mechanisms to give the desired effect.

A simple, specific, accurate, precise and affordable high performance liquid chromatographic method for the simultaneous determination of antihypertensive drug combination consisting of a calcium channel blocker-amlodipine, an angiotensin II receptor antagonist-valsartan and a thiazide diuretic-hydrochlorothiazide was developed and validated.

The mobile phase systems consisted of varying mixtures of acetonitrile, distilled water and phosphate buffer adjusted to the required pH with orthophosphoric acid. The mobile phases were degassed by ultrasonication before use. The optimized conditions for the separation of the 3 analytes amlodipine, valsartan and hydrochlorothiazide consisted of Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm) column, mobile phase: acetonitrile-potassium dihydrogen phosphate pH 3.0-water, (75:6:19, % v/v/v). Column temperature was maintained at 40 ^{o}C; Flow rate: 1 ml/min; detection: 229 nm; and injection of 20 µl. The samples were dissolved in mobile phase for optimal chromatographic parameters.

The precision of the method was shown through adequate repeatability or intraday precision (CV ≤ 2) and interday precision (CV ≤ 2). The method demonstrated adequate linearity of detector response over the range of 25-150%. The linearity equations were y = 4537 x + 26628, R^2 = 0.991 for hydrochlorothiazide, y = 5344 x + 124106, R^2 = 0.997 for valsartan and y = 4227x + 9893, R^2 = 0.995 for amlodipine. The limit of detection for hydrochlorothiazide, valsartan and amlodipine were 10.72, 21.20 and14.45 ng, while the limits of quantification were 35.76, 71.23 and 48.16 ng respectively. The method also showed adequate robustness to small variations in mobile phase pH, column temperature and acetonitrile concentrations. The full recoveries of each working standard for all compounds were within ICH specifications of 98-103% which showed that the method was accurate.

The developed method is rapid (run time 6 min), selective, requires simple sample preparation procedures and simple mobile phase combinations. It is also cost effective and represents a good procedure for determination of hydrochlorothiazide, valsartan and amlodipine in bulk raw materials and pharmaceutical dosage forms.

The method was applied in the assay of 6 commercial products containing all the three, combination of any two or any one of the three API obtained from randomly selected retail pharmacies located within the Thika town. Three batches of each product were analyzed. The assay results indicated that there was no significant inter-batch variation in the content of active ingredients in the products tested except in two. The most noticeable feature was the high content in most of samples of the amlodipine component whose assay value was found to be more than 110% with content as high as 129.6%. For all the commercial samples tested only 2 batches complied with the assay limits defined in the B.P. and U.S.P.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Physiological regulation of blood pressure

Blood pressure is the pressure of the blood within the arteries produced primarily by the contraction of the heart muscle. The first (systolic pressure) is measured after the heart contracts and is high while the second (diastolic pressure) is measured before the heart contracts and is low. It varies with the strength of the heartbeat, the elasticity of the arterial walls, the volume and viscosity of the blood and a person's health, age, and physical condition. The arterial blood pressure (BP) is directly proportional to the cardiac output (CO) and peripheral vascular resistance (PVR).

Physiologically, in both normal and hypertensive individuals, blood pressure is maintained by moment-to-moment regulation of cardiac output and peripheral vascular resistance, exerted at three anatomic sites namely; arterioles, post capillary venules (capacitance vessels) and heart. A fourth anatomic control site, the kidney, contributes to maintenance of blood pressure by regulating the volume of intravascular fluid. Baroreflexes, mediated by autonomic nerves, act in combination with humoral mechanisms, including the renin-angiotensin-aldosterone system, to coordinate function at these four control sites and thus maintain normal blood pressure [1, 2].

Hypertension or high blood pressure, sometimes called arterial hypertension, is a chronic medical condition in which the blood pressure in the arteries is elevated. This requires the heart to work harder than normal to circulate blood through the blood vessels. Blood pressure is assessed by two measurements, systolic and diastolic, which depend on whether the heart muscle is contracting (systole) or relaxed between beats (diastole). Normal blood pressure at rest is within the range of 100-140 mmHg systolic and 60-90 mmHg diastolic. High blood pressure is diagnosed as first stage if the blood pressure is persistently at or above 140/90 mmHg or as second stage if it’s persistently above 160/100 mmHg.

Hypertension is classified as either primary (essential) hypertension or secondary hypertension; about 90 -95% of cases are categorized as primary hypertension which means high blood

pressure with no obvious underlying medical cause. The remaining 5-10% of cases (secondary hypertension) is caused by other conditions that affect the kidneys, arteries, heart or endocrine system [1].

1.2 Etiology of hypertension

The pathogenesis of essential hypertension is multi-factorial with genetic factors playing an important role. People whose one or both parents are hypertensive have high chances of getting hypertenson. Environmental factors are also significant contributors such as increased salt intake and obesity. These factors alone are probably not sufficient to raise blood pressure to abnormal levels but are synergistic with a genetic predisposition [1].

Other factors that may be involved in the pathogenesis of essential hypertension are sympathetic nervous system hyperactivity, abnormal cardiovascular development, renin-angiotensin activity and defect in natriuresis, intracellular sodium and calcium, as well as exacerbating factors such as alcohol, cigarette smoking and polycythemia [2].

In most cases, elevated blood pressure is associated with an overall increase in resistance to flow of blood through arterioles, whereas cardiac output is usually normal. Meticulous investigation of autonomic nervous system function, baroreceptor reflexes, the renin-angiotensin-aldosterone system and the kidney have failed to identify a single abnormality as the cause of increased peripheral vascular resistance in essential hypertension. The heritability of essential hypertension is estimated to be about 30%. Mutations in several genes have been linked to various rare causes of hypertension. Functional variations of the genes for angiotensinogen, angiotensin-converting enzyme (ACE), the adrenoceptors and adducin (a cytoskeletal protein) appear to contribute to some cases of essential hypertension [1].

Sustained arterial hypertension damages blood vessels in the kidney, heart and brain thus leading to an increased incidence of renal failure, coronary disease, heart failure and stroke. Effective pharmacological lowering of blood pressure has been shown to prevent damage to blood vessels and to substantially reduce morbidity and mortality rates [1].

1.3 Prevalence and incidence of the hypertension

Essential hypertension applies to the 95% of cases in which no cause for hypertension can be identified. This occurs in 10-15% of white adults and 20-30% of black adults in the United States of America. The onset is usually between 25-55 age brackets and it is uncommon before the age of 20 years. Secondary hypertension should be suspected in children or young adults and in older persons in whom onset of hypertension is new or in whom hypertension suddenly worsens.

Hypertension is the leading cause of cardiovascular disease worldwide. Prior to 1990, population data indicated that hypertension prevalence was decreasing. However, recent data suggested that it is again on the rise. In 1999-2000, 28.7% of National Health and Nutrition Survey (USA) participants had hypertension, an increase of 3.7% from 1988-1991. Hypertension prevalence was highest in non-Hispanic blacks (33.5%), increased with age (65.4% among those aged $\geq$ 60 years), and tended to be higher in women (30.1%). In a multiple regression analysis, increasing age, increasing body mass index and non-Hispanic black race/ethnicity were independently associated with increased rates of hypertension. In 1999-2000, 68.9% were aware of their hypertension and hypertension was controlled in 31.0%. Women, Mexican Americans, and those aged 60 years or older had significantly lower rates of control compared with men, younger individuals and non-Hispanic whites [3].

The incidence of hypertension range between 3% and 18%, depending on the age, gender, ethnicity and body size of the population studied. Despite advances in hypertension treatment, the control rates continue to be suboptimal and only about one third of all hypertensives are controlled in the United States of America [3, 4].

In a study conducted in the slums of Nairobi Kenya, 19.1% of the population was hypertensive with significant higher outcomes among females, older people and those of Kamba ethnicity. The overall awareness among hypertensives was 21% which was lower in males, young adults and those having informal jobs. Among those who were hypertensive 15.1% were on treatment with significant more females and elderly. Among the hypertensive patients on treatment 5.1% had their blood pressure controlled and this was significantly higher in females [5].

Unfortunately, several surveys indicate that only a third to a half of people with hypertension have adequate blood pressure control despite the availability of several potentially effective

drugs. Knowledge of the drugs' antihypertensive mechanisms and sites of action of the drugs allows accurate prediction of efficacy and toxicity. As a result, rational use of these agents, alone or in combination, can lower blood pressure with minimal risk of serious toxicity in most patients [1].

1.4 Pharmacology of antihypertensive agents

All antihypertensive agents act at one or more of the four anatomic control sites and produce their effects by interfering with normal mechanisms of blood pressure regulation. Classification of these agents is done according to the principal regulatory site or mechanism by which they act. Some of these antihypertensives are also used to treat heart failure for example hydrochlorothiazide, furosemide, spironolactone, metoprolol, captopril and losartan among others. The categories are summarized in table 1.1 [1, 6].

Table 1.1: Drugs used in hypertension [1]

Class	Sub class	Examples	Mechanism of action
Diuretics	Thiazides:	HCTZ	Block Na/Cl ion transporter in renal distal convoluted tubule
	Loop diuretics:	Furosemide	Block Na/K/Cl ion transporter in renal loop of Henle
	Aldosterone antagonist	Spironolactone & Eplerenone	Block aldosterone receptor in renal collecting tubule
Sympathoplegics	Centrally acting	Clonidine,	Activate α 2 adrenoceptors
		Methydopa	
Sympathetic nerve terminal blockers	α Blockers	Prazosin	Selectively block α1 adrenoceptor
	β Blockers	Metoprolol	Block β receptors
Vasodilators		Amlodipine	Blocks cardiac & vascular Ca channels
		Hydralazine	Causes nitric oxide release
Parenteral agents		Nitroprusside	Releases nitric oxide
Angiotensin-converting enzyme(ACE)inhibitors		Captopril Lisinopril	Inhibit angiotensin converting enzyme
Angiotensin receptor blockers		Losartan Valsartan	Block AT1 angiotensin receptors
Renin inhibitor		Aliskiren	Inhibits enzyme activity of renin

1.5 Multi-component antihypertensive drugs

Drug monotherapy in hypertension management is desirable because compliance is likely to be better, there is lower overall cost and fewer adverse effects. However, most patients with hypertension require two or more drugs, preferably acting by different mechanisms to give the desired effect.

Studies have shown that up to 40% of patients may respond inadequately even to two agents and are considered to have "resistant hypertension". Some of these patients have undetected treatable secondary hypertension. Most do not respond and therefore three or more drugs are required [1].

One rationale for polypharmacy in hypertension is that most drugs evoke compensatory regulatory mechanisms for maintaining blood pressure. For example, vasodilators such as hydralazine cause a significant decrease in peripheral vascular resistance, but evoke a strong compensatory tachycardia and salt and water retention that is capable of reversing their effect. The addition of a beta blocker prevents the tachycardia while the addition of a diuretic like hydrochlorothiazide prevents the salt and water retention. In effect, all three drugs increase the sensitivity of the cardiovascular system to their actions.

A second argument for polypharmacy in hypertension is that some drugs have only modest efficacy but reduction of long-term morbidity mandates their use. Many studies on angiotensin-converting enzyme inhibitors report a maximal lowering of blood pressure of less than 10 mm Hg. In patients with stage 2 hypertension (pressure >160/100 mm Hg) this is inadequate to prevent all the sequelae of hypertension, but ACE inhibitors have important long-term benefits in preventing or reducing renal disease in diabetic persons and reduction of heart failure [1].

In addition, the toxicity of some drugs prevents their use at the effective dosage. The widespread indiscriminate use of beta blockers has been criticized because several large clinical trials indicate that some drugs in this group like metoprolol and carvedilol have a greater benefit than others such as atenolol. However, all beta blockers appear to have similar benefits in reducing mortality after myocardial infarction, so these drugs are particularly indicated in patients with myocardial infarction and hypertension. In practice, when hypertension does not respond adequately to a regimen of one drug, a second drug from a different class with a different

mechanism of action and different pattern of toxicity is added. If the response is still inadequate and compliance is known to be good, a third drug should be added [1].

1.6 Treatment of hypertension

1.6.1 Non-pharmacological control of hypertension

Dietary sodium restriction has been known for many years to decrease blood pressure in hypertensive patients. With the advent of diuretics, sodium restriction was thought to be less important. However, there is now general agreement that dietary control of blood pressure is a relatively nontoxic therapeutic measure and may be preventive. Even modest dietary sodium restriction lowers blood pressure in many hypertensive persons. A reduction in dietary salt intake by 9.5% could prevent a million deaths from stroke and myocardial infarction and reduce health care costs [7]. Treatment of hypertension depends upon severity and response to interventions. Mild hypertension may respond to life style changes with diet and exercise. Dietary changes include reducing intake of sodium and increasing intake of fruits and vegetables. Weight reduction to a Body Mass Index of less than 25 is beneficial [8].

1.6.2 Diuretics

Diuretics lower blood pressure primarily by depleting body sodium stores. Sodium is believed to contribute to vascular resistance by increasing vessel stiffness and neuronal reactivity, possibly related to altered sodium-calcium exchange with a resultant increase in intracellular calcium. These effects can be reversed by diuretics or sodium restriction.

In more severe hypertension, diuretics are used in combination with sympathoplegic and vasodilator drugs to control the tendency toward sodium retention caused by these agents. Vascular responsiveness is diminished by sympathoplegic and vasodilator drugs, so that the vasculature behaves like an inflexible tube. Thiazide diuretics such as hydrochlorothiazide are appropriate for most patients with mild or moderate hypertension and normal renal and cardiac function. Potassium-sparing diuretics are useful both to avoid excessive potassium depletion and to enhance the natriuretic effects of other diuretics [1].

1.6.3 Calcium channel blockers

In addition to their antianginal and antiarrhythmic effects calcium channel blockers also reduce peripheral resistance and blood pressure. The mechanism of action in hypertension and partly in angina is the inhibition of calcium influx into arterial smooth muscle cells. Verapamil, diltiazem and the dihydropyridine family (amlodipine, felodipine, isradipine, nicardipine, nifedipine and nisoldipine) are all equally effective in lowering blood pressure. Clevidipine is a newer member of this group that is formulated for intravenous use only.

Amlodipine is the most widely prescribed drug for hypertension and its class was recommended as first-line treatment by the UK's National Institute for Health and Clinical Excellence in recent draft guidance. Patients starting amlodipine will have better controlled hypertension at all times and be less likely to have adverse events if amlodipine is prescribed in combination with aliskiren from the start of treatment.

Hemodynamic differences among calcium channel blockers may influence the choice of a particular agent. Nifedipine and the other dihydropyridine agents are more selective as vasodilators and have less cardiac depressant effect than verapamil and diltiazem. Reflex sympathetic activation with slight tachycardia maintains or increases cardiac output in most patients given dihydropyridine. Verapamil has the greatest depressant effect on the heart and may decrease heart rate and cardiac output. Diltiazem has intermediate actions [9].

1.6.4 Inhibitors of angiotensin.

Renin, angiotensin and aldosterone play important roles in some patients with essential hypertension. Blood pressure of patients with high-renin hypertension responds well to drugs that interfere with the system, supporting a role for excess renin and angiotensin in this population. Renin release from the kidney cortex is stimulated by reduced renal arterial pressure, sympathetic neural stimulation and reduced sodium delivery or increased sodium concentration at the distal renal tubule. Renin acts upon angiotensinogen to split off the inactive precursor to the decapeptide angiotensin I. Angiotensin I is then converted, primarily by endothelial angiotensin converting enzyme to the arterial vasoconstrictor octapeptide angiotensin II which is in turn converted in the adrenal gland to angiotensin III. Angiotensin II possesses vasoconstrictor and sodium-retaining activities while Angiotensin II and III both stimulate aldosterone release. A

parallel system for angiotensin generation exists in several other tissues like the heart and may be responsible for trophic changes such as cardiac hypertrophy. The angiotensin converting enzyme involved in tissue angiotensin II synthesis is also inhibited by ACE inhibitors.

Three classes of drugs act specifically on the renin-angiotensin system. Angiotensin-converting enzyme inhibitors are competitive inhibitors of angiotensin at its receptors. They include losartan and other nonpeptide antagonists and aliskiren, an orally active renin antagonist. Angiotensin-converting enzyme inhibitors like captopril inhibit the angiotensin converting enzyme peptidyldipeptidase that hydrolyzes angiotensin I to angiotensin II. Enalapril is an oral prodrug that is converted by hydrolysis to an angiotensin converting enzyme inhibitor, enalaprilat, with effects similar to those of captopril. Lisinopril is a lysine derivative of enalaprilat. Benazepril, fosinopril, moexipril, perindopril, quinapril, ramipril and trandolapril are other long-acting member of this class. All are prodrugs which are converted to the active agents by metabolizes, primarily in the liver. Angiotensin converting enzyme inhibitors have a particularly useful role in treating patients with chronic kidney disease because they diminish proteinuria and stabilize renal function. ACE inhibitors have also proved to be useful in the treatment of heart failure after myocardial infarction. There is recent evidence that ACE inhibitors reduce the incidence of diabetes in patients with high cardiovascular risk [1].

The angiotensin receptor blocking agents losartan and valsartan were the first marketed blockers of the angiotensin II type 1 receptor. Candesartan, eprosartan, irbesartan, telmisartan and olmesartan have no effect on bradykinin metabolism and are therefore more selective blockers of angiotensin effects than ACE inhibitors. They also have the potential for more complete inhibition of angiotensin action compared with ACE inhibitors because there are enzymes other than ACE that are capable of generating angiotensin II. Angiotensin receptor blockers provide benefits similar to those of ACE inhibitors in patients with heart failure and chronic kidney disease [1].

1.7 Chemistry of the drugs under study

1.7.1 Introduction

The chemistry of drugs molecules determines how they are absorbed from the site of administration and how they are transported to and concentrated at the site of action. The functional groups of a drug influence various physicochemical properties that influence drug action, water and lipid solubility, ionization, chemical bonding, stereochemistry, surface activity, redox potential and polymorphism. Chemistry of drugs plays an important role in analysis of pharmaceuticals, which forms the basis of drug quality control. Methods involving spectroscopic, chromatographic, potentiometric and titrimetric techniques are all dependent on the chemistry of the drug. The applications of these techniques in pharmacy are stressed in analysis of pharmaceuticals, drug development and drug quality.

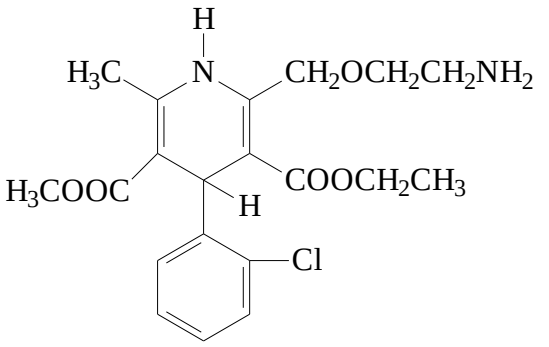
1.7.2 Valsartan

Valsartan is a white fine powder. It is soluble in ethanol and methanol but slightly soluble in water. Its melting point is 117 ^{o}C. Valsartan is available as tablets for oral administration, containing 40, 80, 160 or 320 mg of the drug. Valsartan is a nonpeptide, orally active and specific angiotensin II receptor blocker acting on the AT_1 receptor subtype. Valsartan (figure 1.1) is chemically described as N-(1-oxopentyl)-N-[[2'-(1H-tetrazol-5-yl) [1, 1'-0biphenyl]-4-yl] methyl]-L-valine. Its empirical formula is $C_{24}H_{29}N_5O_3$ with a molecular weight of 435.5.

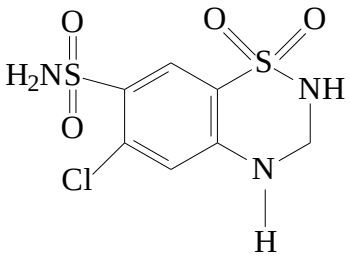
Valsartan is a free acid with two acidic hydrogen atoms (pK_a 3.9 and 4.7): the hydrogen atom of the carboxyl group and that of the tetrazole ring. Accordingly, both acidic hydrogens atoms can form salts with a base. The free acid valsartan has a melting point in a closed crucible of 80-95 ^{o}C and in an open crucible of 105-110 ^{o}C and a melting enthalpy of 12 k J/mol. The specific optical rotation of valsartan in 1% w/v in methanol is $\{[\alpha]_D^{20} = (-70 \pm 2)^0\}$. The free acid is characterized as amorphous under X-ray crystallographic techniques. The melting point linked with the measured melting enthalpy confirms the existence of a considerable residual arrangement in the particles or structural domains for the free acid valsartan [10].

1.7.3 Amlodipine

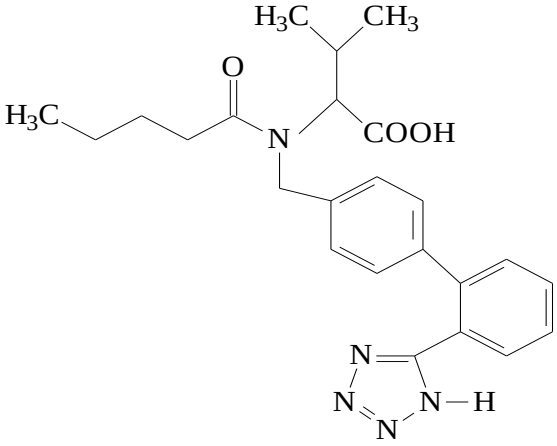
Amlodipine besylate is a white crystalline powder with a molecular weight of 567. It is slightly soluble in water and sparingly soluble in ethanol (pK_a 8.6). Its empirical formula is $C_{20}H_{25}ClN_2O_5 \bullet C_6H_6O_3S$ (figure 1.1). Amlodipine besylate is chemically described as 3-Ethyl-5-methyl (±)-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-l, 4-dihydro-6-methyl-3, 5-pyridinedicarboxylate, monobenzenesulphonate. Amlodipine besylate tablets are formulated as white tablets equivalent to 2.5, 5, and 10 mg of amlodipine for oral administration. Amlodipine is formulated as the besylate salt of amlodipine, a long-acting calcium channel blocker [11].



Amlodipine



Hydrochlorothiazide



Valsartan

Figure 1.1: Chemical structures of amlodipine, valsartan and hydrochlorothiazide

1.7.4 Hydrochlorothiazide

Hydrochlorothiazide is a white, practically odorless crystalline powder, slightly soluble in water, sparingly soluble in alcohol and soluble in acetone (pK_a 7.0). Hydrochlorothiazide dissolves in dilute solutions of alkali hydroxides. It is insoluble in chloroform, in ether and in dilute mineral acids; freely soluble in dimethylformamide, in n-butyl amine. It has an empirical formula $C_7H_8ClN_3O_4S_2$, molecular weight of 297.7 (figure 1.1). Hydrochlorothiazide is chemically described as 6-chloro-1,1-dioxo-3, 4-dihydro-2*H*-1, 2, 4-benzothiadiazine-7-sulfonamide [12-14].

1.8 Assay methods for the antihypertensive drugs under study

1.8.1 Valsartan

A spectrophotometric method was developed for simultaneous determination of amlodipine and valsartan. In this method amlodipine in methanolic solution was determined using zero order UV spectrophotometry by measuring its absorbance at 360.5 nm without interference from valsartan. The valsartan spectrum in zero order was totally overlapped with that of amlodipine. First, second and third derivative could not resolve the overlapped peaks. The first derivative of the ratio spectra technique was applied for the measurement of valsartan. The limit of quantification of amlodipine and valsartan were 10-80 μg/ml and 20-180 μg/ml, respectively. The method was successfully applied for the quantitative determination of both drugs in bulk powder and pharmaceutical formulation [15].

A rapid, sensitive and accurate liquid chromatographic-tandem mass spectrometry method was described for the simultaneous determination of nebivolol and valsartan. The drugs were extracted from plasma using acetonitrile and separated on a C-18 column. For this purpose a mobile phase consisting of acetonitrile: 0.05 mM formic acid pH 3.5 (50:50, % v/v) delivered at a flow rate of 0.25 ml/min was used [16].

Isocratic reversed-phase high performance liquid chromatography method was developed for determination of valsartan in tablet dosage form. The method was carried out using Thermo-Hypersil

C-18 (150 mm × 4.6 mm i.d. 5 µm) with mobile phase consisting of water: acetonitrile: glacial acetic acid (500:500:01, v/v/v). The flow rate was set at 1.0 ml/min and effluent was detected at 273 nm [17].

High performance thin layer chromatographic method was established for simultaneous analysis of valsartan and hydrochlorothiazide in tablet formulations. Standard and sample solutions of valsartan and hydrochlorothiazide were applied to pre-coated silica gel G 60 F254 HPTLC plates and the plates were developed with chloroform: ethyl acetate: acetic acid, (5:5:0.2, v/v/v) as the mobile phase. UV detection was performed densitometrically at 248 nm [18].

A stability-indicating HPLC assay method was developed and validated for valsartan in bulk drug and pharmaceutical dosage forms. An isocratic reverse phase HPLC was achieved using Symmetry C-18 (250 mm × 4.6 mm i.d. 5 µm) column with the mobile phase consisting of 0.02 mM sodium phosphate buffer pH 2.5 and acetonitrile in the ratio of (58:42, %v/v). The stress testing of valsartan was carried out under acidic, alkaline, oxidative thermal and photolytic conditions. Valsartan was well resolved from its degradation products [19].

A stability-indicating gradient reversed-phase ultra performance liquid chromatographic method was developed for the quantitative determination of the purity of valsartan drug substance and drug products in bulk samples and pharmaceutical dosage forms in the presence of its impurities and degradation products. The method was developed using Waters C-18 (100 mm × 2.1 mm i.d. 1.7 µm) column. The eluted compounds were monitored at 225 nm [20].

A HPLC method using a monolithic column was developed for the determination of valsartan in human plasma. The assay is based on protein precipitation using acetonitrile and fluorescence detection. The assay enables the measurement of valsartan for therapeutic drug monitoring with a minimum limit of quantification of 20 ng/ml. The separation was carried out in reversed-phase conditions using a Chromolith Performance C-18 (100 mm × 4.6 mm i.d.5 µm) column with an isocratic mobile phase consisting of 0.01 M disodium hydrogen phosphate buffer pH 3.5: acetonitrile (60:40, % v/v). The excitation and emission wavelengths were set at 230 and 295 nm, respectively [21].

Official pharmacopoeia (USP 2007) contain monographs in which valsartan as raw material and in finished products may be assayed by chromatography. The chromatographic system consists of C-18 (250 mm × 3 mm i.d. 5 µm) column equipped with 273 nm detector. The mobile phase consists of water: acetonitrile: glacial acetic acid (500:500:1, v/v/v) pumped at a flow rate of 0.4 ml/min [14].

1.8.2 Amlodipine

A sensitive enantioselective gas chromatographic assay was developed for amlodipine. The assay involves conversion of the (+)-(*R*)- and (−)-(*S*)-enantiomers of amlodipine into their acyl derivatives with the chiral reagent (+)-(*S*)-α-methoxy-α-trifluoromethylphenylacetyl chloride (Mosher's reagent). Peak separation after chromatography of the diastereomers was greater than 85 % and the lower limit of detection in blood plasma was 0.02 ng/ml for each enantiomer. The method was used for the measurement of amlodipine enantiomers in human, rat and dog plasma and in other organs of the rat [22].

Similarly, two simple spectrophotometric methods for simultaneous determination of amlodipine besylate and nebivolol hydrochloride in tablet formulation were reported. The first method was absorbance correction method based on determination of amlodipine besylate at 365 nm using its absorptivity value and nebivolol hydrochloride at 280 nm after deduction of absorbance due to amlodipine besylate. The second method is based on absorbance ratio in which the wavelengths selected were 269 nm, an isoabsorptive point and 280 nm as λ_{max} of nebivolol hydrochloride. The methods can be routinely adopted for quality control of these drugs in tablets [23].

A reversed-phase liquid chromatography assay with UV detection for the assay of amlodipine was developed. The method utilized a C-18 column (250 mm × 4.6 mm i.d. 5 µm) with a mobile phase composed of a mixture of methanol: 0.04 M ammonium acetate: acetonitrile (38:38:24, % v/v/v) containing 0.02 % triethylamine pH 7.1 [24].

An isocratic reversed-phase stability-indicating HPLC method was developed and validated for the simultaneous determination of atorvastatin and amlodipine in commercial tablets. The method showed adequate separation for amlodipine, atorvastatin from their associated main

impurities and their degradation products. Separation was achieved on a Perfectsil ODS-3, (250 mm × 4.6 mm i.d. 5 µm) column using a mobile phase consisting of acetonitrile: 0.025 M NaH_2PO_4 buffer pH 4.5 (55:45, % v/v) at a flow rate of 1 ml/min and UV detection at 237 nm [25].

A reverse-phase high-performance liquid chromatographic method was developed for the simultaneous determination of atorvastatin calcium and amlodipine besylate in tablet dosage forms. A Phenomenex Luna C-18 (250 mm × 4.6 mm i.d. 5 µm) in isocratic mode, with mobile phase containing methanol: acetonitrile: 50 mM KH_2PO_4 pH 3.5 (20:50:30, % v/v/v) was used. The flow rate was 1.0 ml/min and effluent was monitored at 240 nm [26].

An isocratic reversed-phase stability-indicating HPLC method was developed and validated for the simultaneous determination of atorvastatin and amlodipine in commercial tablets. The method has shown adequate separation for amlodipine and atovarstatin from their associated main impurities and their degradation products. Separation was achieved on a Perfectsil ODS-3 (250 mm × 4.6 mm i.d. 5 µm) column using a mobile phase consisting of acetonitrile: 0.025 M NaH_2PO_4 buffer pH 4.5 (55:45, % v/v) at a flow rate of 1 ml/min and UV detection at 237 nm. The drugs were subjected to oxidation, hydrolysis, photolysis and heat to apply stress conditions [27].

A simple, rapid, sensitive and stability indicating high performance liquid chromatography assay method for simultaneous determination of amlodipine besylate and valsartan in bulk and commercial formulation was developed. Analytical separation was achieved with Waters Symmetry C-18 Column (150 mm × 4.6 mm) using a combination of methanol and 0.01 M potassium dihydrogen phosphate buffer pH 2.5 (60:40 v/v), with flow rate of 1 ml/ min.

The retention times for amlodipine and valsartan were found to be 4.6 and 7.6 min, respectively. Both the drugs were exposed to thermal, photolytic, hydrolytic and oxidative stress conditions individually as well as in combination, subsequently samples were analyzed by the proposed method. Detection was done using photodiode array detector at 238 nm. Method was found to be very sensitive as limit of detection was found to be 20 ng/ml and 44 ng/ml for amlodipine

besylate and valsartan, respectively. The method was found to be specific and stability indicating as no interfering peaks of degradation compounds and excipients were noticed [28].

Official pharmacopoeia contains monographs in which amlodipine as raw material and in finished products may be assayed by liquid chromatography with UV detection at 237 nm. Assay of amlodipine is done by liquid chromatography which is done for the test and the standard. The percentage content of amlodipine besylate is calculated from the areas of the peaks and the declared content in amlodipine besylate in chemical reference substances. Amlodipine should be stored in an airtight container, protected from light [14].

1.8.3 Hydrochlorothiazide

A high performance liquid chromatographic procedure was reported for the determination of hydrochlorothiazide (HCTZ) and enalapril in pharmaceutical tablets. An aliquot of the sample was dissolved in 15% acetonitrile containing theophylline as an internal standard and chromatographed on a Supelcosil LC-8 (150 mm × 4.6 mm i.d. 5 µm) column. The mobile phase was 3.0 mM tetrabutyl ammonium hydrogen sulfate in acetonitrile: water: triethylamine (14, 85.6, 6.4, % v/v) adjusted to pH 4.1 using glacial acetic acid. The detection was at 220 nm [29].

A sensitive and specific HPLC method was developed and validated for the simultaneous determination of enalapril and hydrochlorothiazide in human plasma using a C-18 reversed-phase column. The average recoveries ranged from 0.005-0.1 µg/ml and 0.01-0.2 µg/ml for each drug and the percentage recoveries were 92.7% and 93.3%, respectively. The limits of detection were 2.5 and 0.14 ng/ml for enalapril and hydrochlorothiazide respectively. The validated method was successfully used to study enalapril and hydrochlorothiazide pharmacokinetics, bioavailability and bioequivalence in 24 adult volunteers [30].

A stability-indicating method for the determination of hydrochlorothiazide in tablet formulations and in the bulk form was described. Hydrochlorothiazide was dissolved or extracted using methanol. The resulting solution, containing sulfadiazine as an internal standard was run on a C-18 (250 mm × 4.6 mm i.d. 10 µm) with an aqueous mobile phase containing 5 % methanol as the organic modifier. The method can also be used as a test for impurities in hydrochlorothiazide [31].

Official pharmacopoeia contains monographs in which hydrochlorothiazide as raw material and in finished products may be assayed by potentiometric method [13] and by liquid chromatography [14, 32]. The chromatographic system consists of (250 mm × 4.6 mm i.d. 3-10 µm) equipped with 254 nm detector. The mobile phase consists of 0.1 M monobasic sodium phosphate pH 3.5: acetonitrile (9:1, v/v) pumped at a flow rate of 2.0 ml/minute.

A reversed-phase HPLC method has been reported for the simultaneous estimation of amlodipine besylate, valsartan and hydrochlorothiazide in pharmaceuticals using Phenomenex Luna C-18 (150 mm × 4.6 mm. i.d. 5 µm). The mobile phase consisting of acetonitrile: methanol: 50 mM phosphate buffers pH 3.0 was pumped at a flow rate of 1.0 ml/min in the ratios of (20:50:30, % v/v/v) and the eluent monitored at 239 nm [33].

Simple, accurate, precise, sensitive and validated HPLC and HPTLC-densitometric methods were developed for simultaneous determination of amlodipine, valsartan and hydrochlorothiazide in combined tablet dosage form. The gradient reversed-phase HPLC analysis was performed on a Phenomenex Luna C-18 (150 mm × 4.6 mm i.d. 5 µm) column, using a mobile phase consisting of 10 mM ammonium acetate buffer pH 6.7 and methanol in solvent gradient elution for 20 min at a flow rate of 1 ml/min. Quantification was carried out using a photodiode array UV detector at 238 nm. The employment of a diode array detector allowed selectivity confirmation by peak purity evaluation [34].

A simple reversed-phase HPLC method for the quantification of valsartan amlodipine and hydrochlorothiazide in human plasma was developed and validated. The three were resolved using a Gemini C-18 column and mobile phase gradient starting from acetonitrile and 10 mmol/L ammonium formate, pH 3.5 by formic acid (20:80, % v/v) to acetonitrile and 10 mmol/L ammonium formate (70: 30, % v/v), over 20 minutes, with a flow rate of 1 ml/min. The samples were purified by protein precipitation and extraction. Telmisartan was used as internal standard [35].

1.9 Study Justification

To determine the quality of pharmaceutical products with the requisite specificity, accuracy and efficacy, it is necessary that quality control laboratories develop and validate methods for routine use. Method development involves designing a simple, accurate and convenient method for the assay of the desired product formulation. Method development takes into account the different ingredients incorporated into the pharmaceutical product and includes test procedures or steps designed to eliminate any possible interference by such ingredients in product analysis.

The need for compliance and convenience of drug delivery has led to various drugs being combined into single dosage formulations. These may be drugs with synergistic effects or drugs that have different mechanisms of actions whose additive action gives the desired effect or relieve to the patient. However most pharmacopoeias specify assay methods for individual active pharmaceutical ingredient (API) as opposed to combined APIs.

Advances in technology have resulted in sophisticated methods of analysis. These methods are characterized by their relatively high level of selectivity and accuracy, allowing them to be used in the assay of pharmaceutical products as well as in detection and quantification of impurities especially of related substances. The most commonly employed technique for routine quality control testing of pharmaceutical products is liquid chromatography which offers the advantages of high selectivity and sensitivity.

Currently multi-component antihypertensive drugs contain combinations of two or more APIs. A combination of thiazides, adrenergic blockers, diuretics calcium channel blockers, angiotensin converting enzyme antagonists and angiotensin II receptor antagonists are commonly co-formulated. Examples of such drugs in the Kenyan market include; atenololol with chlorthalidone, candesartan with hydrochlorothiazide, candesartan with hydrochlorothiazide, amlodipine with valsartan, amlodipine with valsartan and hydrochlorothiazide, perindopril with indapamide, valsartan with hydrochlorothiazide, losartan with hydrochlorothiazide, perindopril with amlodipine, amlodipine with losartan, irbesartan with hydrochlorothiazide, losartan with hydrochlorothiazide, nifedipine with atenololol and lisinopril with hydrochlorothiazide.

The World Health Organization guidelines on current Good Manufacturing Practices (GMP) specify that the role of ensuring the quality of pharmaceutical products is the responsibility of the Quality Assurance department of any pharmaceutical manufacturing setup. The danger poised to the patient if these combinations do not meet the required standards cannot be underestimated and this raises concerns over the need to have them properly tested and evaluated to ensure that they are of good quality. Developing validated methods that would simultaneously and rapidly determine the quality of these compounds would therefore be a welcome relieve to those charged with the responsibility of ensuring marketed drugs are of quality.

Not much attention has been put on the market surveillance of antihypertensive drugs though hypertension is a major cause of morbidity and mortality. The main focus has been on anti-infective agents such as antibiotics and anti-malarial. There is therefore the need to develop suitable methods of quality control to support the needed market surveillance.

An analytical technique that can simultaneously analyze many components API in a drug product is preferred to those where these drugs are analyzed individually. Analytical methods for drugs in a combination may not be available. If available the methods might not be official in any pharmacopoeias and might be too expensive to be routinely carried out in laboratories. They may also involve cumbersome extraction and separation procedures.

In the reported method [32] the column used was C-18 (150 mm × 4.6 mm i.d. 5 µm) which is not the routine practice. The method also utilizes two organic modifiers with the last component eluting within 9 minutes. The order of elution of the component was hydrochlorothiazide, amlodipine and valsartan and detection was done at 239 nm. In the other reported method [33] a column of similar size was used, the order of elution was hydrochlorothiazide, valsartan and amlodipine respectively with the last to elute at 10.2 min. Detection was done at 238 nm. The aim of this study was to determine the optimum retention time and resolution of amlodipine, valsartan and hydrochlorothiazide using isocratic reversed phase HPLC method, by use of one organic modifier. The column to be used was C-18 (250 mm × 4.6 mm i.d. 5 µm) because these types of columns are the ones that are routinely used in liquid chromatography analysis of

pharmaceuticals though even other columns would be tested. The wavelength of detection would also be optimized.

1.10 Study objectives

The general objective of the study was to develop and validate an isocratic reversed phase liquid chromatographic method for the simultaneous determination of amlodipine, valsartan and hydrochlorothiazide.

The specific objectives were**:**

a. To develop and optimize a high performance liquid chromatographic method with ultraviolet detection for the assay of amlodipine, valsartan and hydrochlorothiazide.

b. To validate the developed high performance liquid chromatographic method method in accordance with International Conference on Harmonization guidelines.

C. To use the developed method in the assay of commercial samples obtained from the Kenyan market containing all the three (amlodipine, valsartan and hydrochlorothiazide), any two or one of the active pharmaceutical ingredients.

CHAPTER TWO

METHODOLOGY

2.1 Introduction

High performance liquid chromatography has become a widely used technique for analysis of many organic substances in large variety of samples. The concentrations to be determined are often in parts per million ranges or less. At these levels the detector characteristics become very important as both sensitivity and selectivity are required [36].

An analytical technique for the simultaneous analysis of the API in a multi-component drug is preferred to analysis of individual drugs. High performance liquid chromatography is the preferred technique used in the simultaneous analysis of several drugs compounds making it cost effective. In this study the focus was to develop a simple, rapid and efficient HPLC method for the simultaneous analysis of hydrochlorothiazide, valsartan and amlodipine, a new antihypertensive drug combination recently introduced in the Kenyan market.

2.2. Materials and reagents.

High performance liquid chromatography grade methanol (Rankem, RFCL Limited, Mumbai, India), HPLC grade acetonitrile (Fisher Scientific, UK Limited, Madison, East Grinstead, UK), orthophosphoric acid (analytical reagent (AR) grade) (May and Baker Ltd, Dagenham, England), potassium dihydrogen phosphate (AR grade) (Loba Chemie, PVT Ltd, Mumbai, India) and freshly laboratory double distilled water were used during method development. The working standards substances of amlodipine and hydrochlorothiazide were from Aurobido Pharma (Mumbai, India) while valsartan was from Ranbaxy Pharma (Mumbai, India).

2.3 Equipment

2.3.1 Liquid chromatographic equipment

The HPLC system consisted of a Cyberlab LC 100 HPLC pump (Cyberlab corporation, Millbury, USA), equipped with universal loop injector, Rheodyne 7725 I (Rheodyne Inc, Cotati, CA, USA) of 20 µl injection capacity and LC 100 UV detector (Wufeng Instruments Co, Shangai, China). The equipment was controlled by a personal computer equipped with WS-100 workstation software (Wufeng Instruments Co, Shangai, China). The compounds were separated on a Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm) column, (*Thermo* Electron Corporation, Waltham, MA, *USA*).

The other types of columns that were also tested included Nucleosil 100 -5 C-18(125 mm × 4 mm i.d. 5 µm) (SMI-LabHut Ltd, Gloucester, GL2 8AX, UK), Phenomenex C-8 (250 mm × 4.6 mm i.d. 5 µm) (Phenomenex Inc, Foster *City*, CA, *USA*) and Capcell Pak C18 (250mm × 4.6 mm i.d. 5 µm) (Shiseido, Tokyo, Japan). The column temperature was controlled using a Lab Tech water bath (Daihan Labtech Co, Mumbai, India).

2.3.2 Melting point apparatus

Determination of the melting points of the working standards was carried out using a liquid heating system using Max digital melting point apparatus (Max Digital Inc, Mumbai, India), calibrated before each determination using World Health Organization (WHO) chemical melting point reference substances vanillin and benzil [13].

2.3.3 Infrared spectrophotometer

The infra-red spectra of the working standards substances were recorded using a Fourier Transformation InfraRed spectrophotometer (FTIR), IR Prestige 21 (Shimadzu, Kyoto, Japan) utilizing IR Solution software Version 1.3. Sample discs were prepared in potassium bromide using a manually operated hydraulic pellet press, Mini Hand Press, MHP 1 (Shimadzu, Kyoto, Japan).

2.3.4 Ultraviolet spectrophotometer

A double beam T90+UV/VIS Spectrophotometer (PG Instrument Ltd, Leicestershire, United Kingdom), supported by UV WIN software 5.2 and quartz cuvettes of 1cm path length was used in obtaining UV spectra of the working standards over the range of 200-400 nm.

2.4 Experimental procedures

2.4.1 Melting point determination

The melting points were determined by filling capillary tubes which had been sealed on one side, half way with each of the drug working standards after the sample powders were dried first for 24 hours [13]. They were heated from 25 ^{o}C to 300 ^{o}C. The temperature ranges at which the compounds melted were recorded.

2.4.2 Infrared spectra

The infra red spectra for the working standards were recorded using 1 mm potassium bromide (KBr) disc containing about 1% w/w of each compound over the range of (4000 to 400) cm^{-1}. The working standards were triturated with potassium bromide then pressed manually to form the discs.

2.4.3 Ultraviolet spectra

About 1 mg of each of the 3 working standards were weighed and dissolved in 10 ml of methanol. This was further diluted to make solutions of 10 µg/ml. A mixture containing 10 µg/ml of each of the three samples was also prepared and scanned in the range of 200-400 nm to determine the optimum wavelength of detection for the simultaneous determination of amlodipine, hydrochlorothiazide, and valsartan.

2.4.4 Preparation of working standard solution

About 12.5 mg of amlodipine, 100 mg of valsartan, and 25 mg of hydrochlorothiazide were accurately weighed and each dissolved in 50 ml acetonitrile. From the stock solution, the mixed

working solution was prepared by pipetting 1ml of each of the stock solutions into the same 25 ml volumetric flask and making up to the volume with acetonitrile-water (50:50, % v/v). The final concentrations of the compounds were 10 µg/ml, 80 µg/ml and 20 µg/ml of amlodipine, valsartan and hydrochlorothiazide, respectively [33]. These concentrations also reflected in approximate the relative proportionate amounts of the 3 drugs in commercial products

CHAPTER 3

METHOD DEVELOPMENT AND OPTIMIZATION

3.1 Melting point determination

The melting points of the working standards were determined and are recorded in table 3.1. The values of the melting points were within ranges reported in literature, indicating identity and purity of the analyte compounds.

Table 3.1: Melting points of the working standards

Compound	Determined melting point (^{o}C)	Literature melting points (^{o}C) [10- 12]
Amlodipine	198-200	199-201
Hydrochlorothiazide.	273-274	274
Valsartan.	117-118	117

3.2 Ultraviolet scan

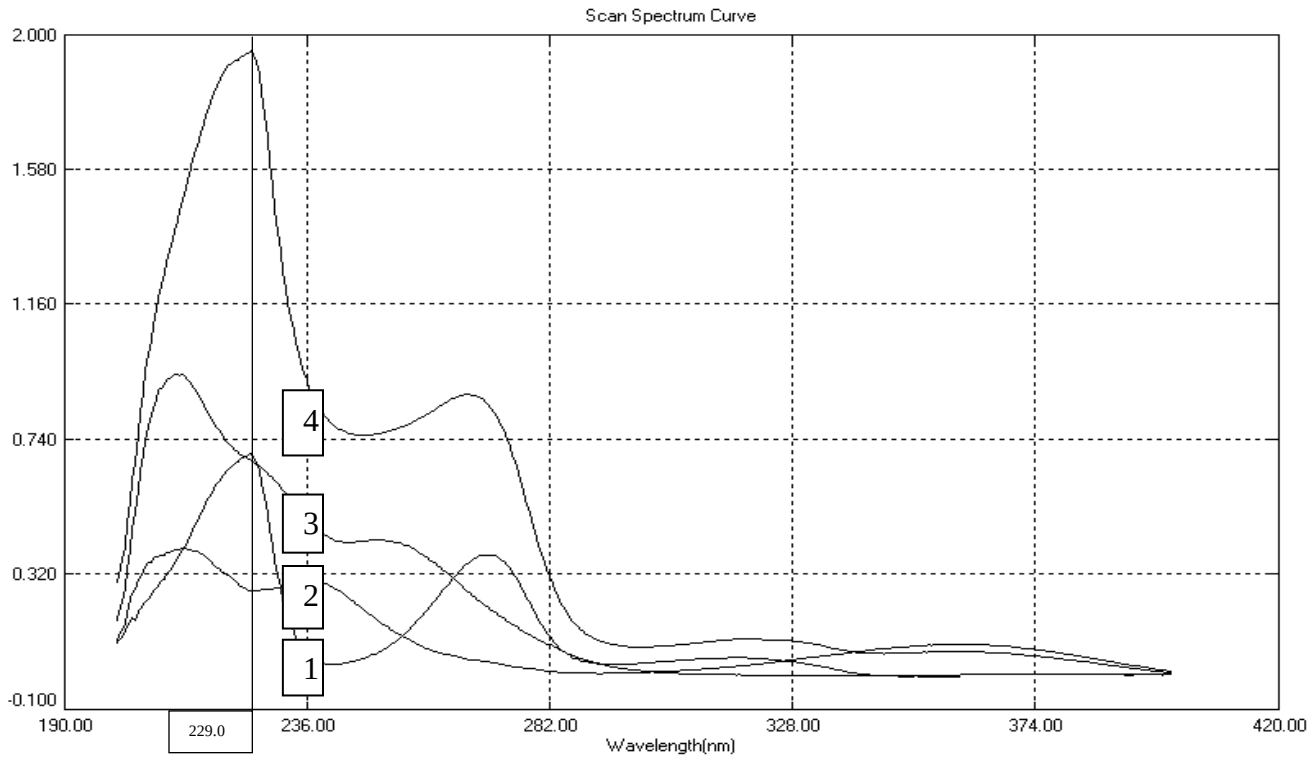
From the overlaid UV spectra the wavelength of 229 nm showed maximum absorption for all the 3 analytes and was chosen for monitoring the analytes during the study. Individual spectra scans were also used for identification purposes (table 3.2 and figure 3.1).

Table 3.2: Absorption maxima of the working standards

Compound	Measured absorption maxima (nm)	Literature absorption maxima (nm) in literature [12]
Amlodipine	220, 239, 365	243,365
Hydrochlorothiazide	225, 270, 315	315
Valsartan	210, 250	250

Samples dissolved in methanol

The observed absorption maxima were similar to those reported in literature confirming the identity of the compounds. The individual spectra are shown in appendices 1-3.



1-Hydrochlorothiazide 2-Amlodipine 3-Valsartan 4-Mixture of the working standards

Figure 3.1: Overlaid ultraviolet spectra of amlodipine, valsartan and hydrochlorothiazide (samples dissolved in methanol)

3.3 Infrared spectra

The frequencies of the principal IR absorption bands were compared against those published in literature to confirm identity (Appendices 4-6). All the spectra were concordant with the corresponding reference spectra [13].

3.4 Stationary phase

From the onset, reversed phase silica based columns were chosen because they offered the advantage of narrow pore size and diameter distribution, high mechanical strength, high column efficiency and superior chromatographic parameters. They are also stable over an extended pH range (2-10) that would allow mobile phase pH manipulation for separation of the components [37].

Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm) was tested first. There was clear resolution of the 3 analytes and chromatographic parameters were within acceptable range. The order of elution was hydrochlorothiazide, valsartan and amlodipine. Phenomenex C-8 (250 mm × 4.6 mm i.d. 5 µm) was tested next. Amlodipine was the first to elute followed by hydrochlorothiazide and then valsartan. Nucleosil 100-5 C-18 (125 mm × 4 mm i.d. 5 µm) and Capcell Pak C-18 (250 mm × 4.6 mm i.d. 5 µm) (250 mm × 4.6 mm i.d. 5 µm) were also tested. There was no clear resolution of the analytes.

Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm) column was chosen for further work since the peaks had good shapes and chromatographic parameters (figure 3.2). The identity of each of the analytes was confirmed by injection of three of the individual working standards separately

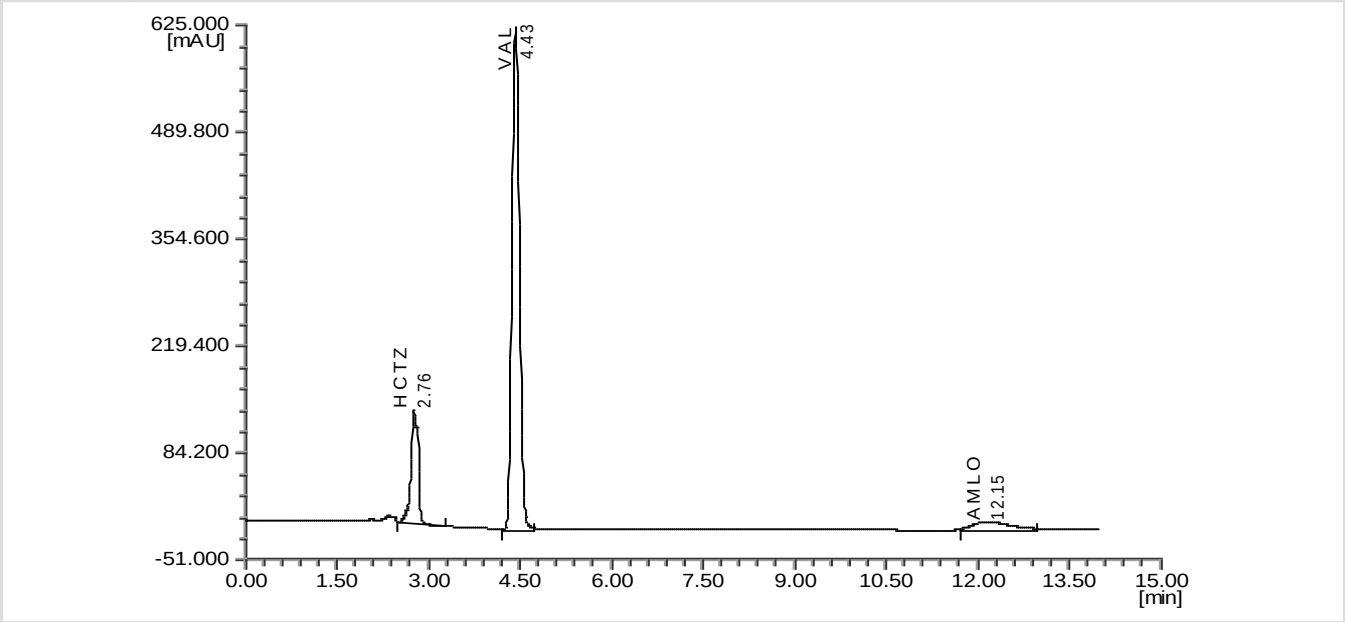


Figure 3.2: Chromatogram showing the selection of the stationary phase for the separation of a mixture of hydrochlorothiazide (HCTZ), valsartan (VAL) and amlodipine (AMLO). Column: Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm). Mobile phase: acetonitrile-0.1 M potassium dihydrogen phosphate pH 2.0-water (60:4:36, % v/v/v). Column temperature: 40 ^{o}C. Flow rate: 1ml/min. Detection: 229 nm. Injection volume: 20 µl.

3.5 Fixed chromatographic conditions

Some chromatographic variables were fixed at the onset of the method development process, based on preliminary observations. These parameters included mobile phase flow rate and sample injection technique and the sample injection amounts. Sample solution of 20 µl was injected. The flow rate for the mobile phase was fixed at 1.0 ml/min. A flow rate of 1.0 ml/min was considered ideal and at no instance during method development did the pressure exceed 100 bars.

Acetonitrile was chosen as the organic modifier solvent to be used in mobile phase preparations because the UV cut off for acetonitrile is 190 nm, allowing lower wavelength of detection. It is also less viscous than methanol, thus causing fewer fluctuations in pressure and its miscible with water and aqueous solutions at all proportions [38]. Preliminary tests also showed poor separation when methanol was used in place of acetonitrile. Arising from the overlaid UV spectra of the analyte compounds a wavelength of 229 nm was chosen for detection of the compounds under study.

3.6 Influence of chromatographic factors

3.6.1 Neutral conditions

Initially a mobile phase consisting of acetonitrile and distilled water (25:75, % v/v) was used for the chromatographic analysis. The column temperature was maintained at 40 ^{o}C and detection was at 229 nm. Under these conditions no separation was achieved among the 3 compounds.

When the mobile phase consisting of acetonitrile and distilled water (50:50, % v/v) was used for the chromatographic analysis and all the other conditions maintained, two of the analyte peaks appeared valsartan and hydrochlorothiazide (figure 3.3). Amlodipine working standard was injected later and its peak signal appeared at 1.88 min indicating that it co-eluted with valsartan.

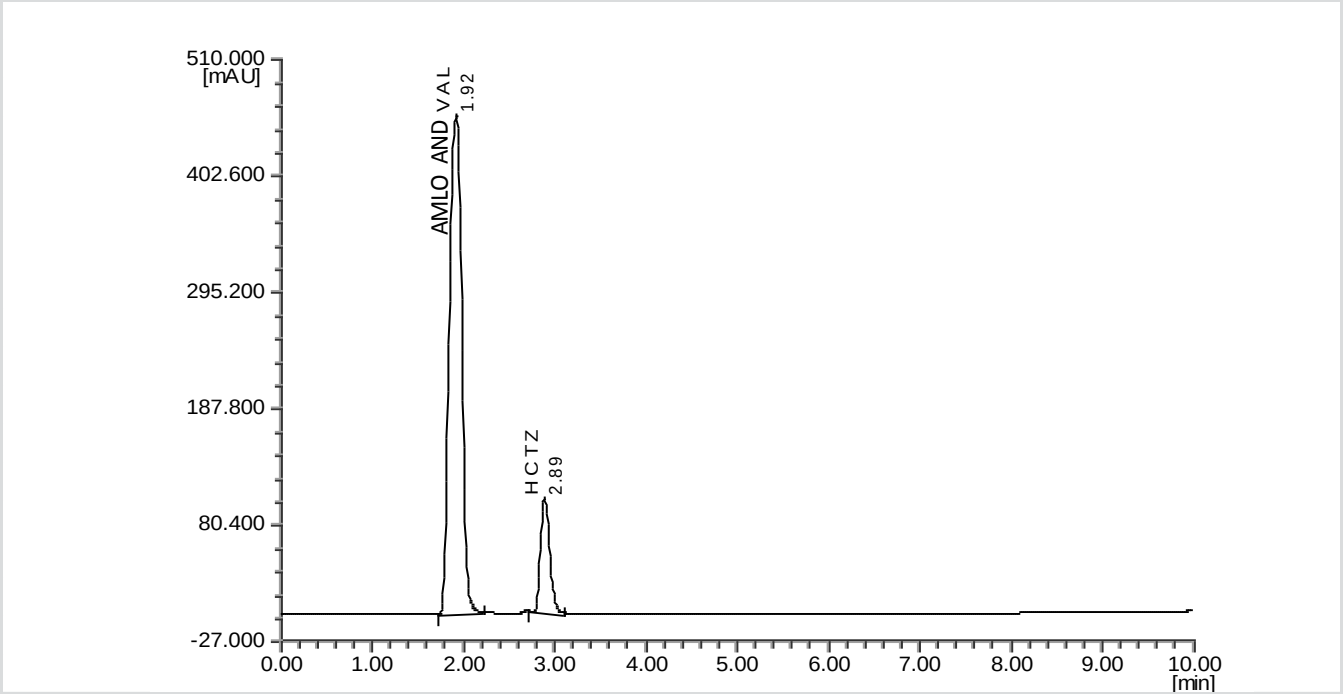


Figure 3.3: Chromatogram showing the effect of unbuffered mobile phase on the separation of a mixture of hydrochlorothiazide (HCTZ), valsartan (VAL) and amlodipine (AMLO). Column: Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm). Mobile phase: acetonitrile-water (50:50, % v/v). Column temperature: 40 ^{o}C. Flow rate: 1ml/min. Detection: 229 nm. Injection volume: 20 µl.

When the mobile phase composition was changed to acetonitrile and distilled water (60:40, % v/v) the peak signals of valsartan and hydrochlorothiazide appeared but they were not resolved. Injection of amlodipine working standard indicated that it had co-eluted with valsartan at 2.42 min. Other mobile combinations with higher amounts of acetonitrile were also tested but did resolve the three analyte compounds.

3.6.2 Effect of different concentrations of acetonitrile pH 4.3

Incorporating an inorganic buffer in the mobile phase was tested to determine its effect on separation compared to that observed using a mobile phase without a buffer. Monobasic potassium dihydrogen phosphate (KH_2PO_4) was selected as the buffer because it is commonly used in reversed-phase liquid chromatography, readily available and has particularly wide buffering capacity that could allow preparation of solutions with pH values ranging over almost the entire pH range (pH 2-13).

The ratios that were tested were (50:4:46, 60:4:36 and 75:4:21, % v/v/v) for acetonitrile: 0.1 M KH_2PO: water, respectively. Among this only acetonitrile-0.1 M KH_2PO_4-water, (60:4:36, % v/v/v) combination ratio showed separation though only two peaks appeared, valsartan and hydrochlorothiazide (figure 3.4). Amlodipine working standard was later injected under similar conditions. Its peak signal appeared at 2.84 min indicating that it had co-eluted with hydrochlorothiazide. The pH of the buffer solution was not adjusted but was found to be 4.3.

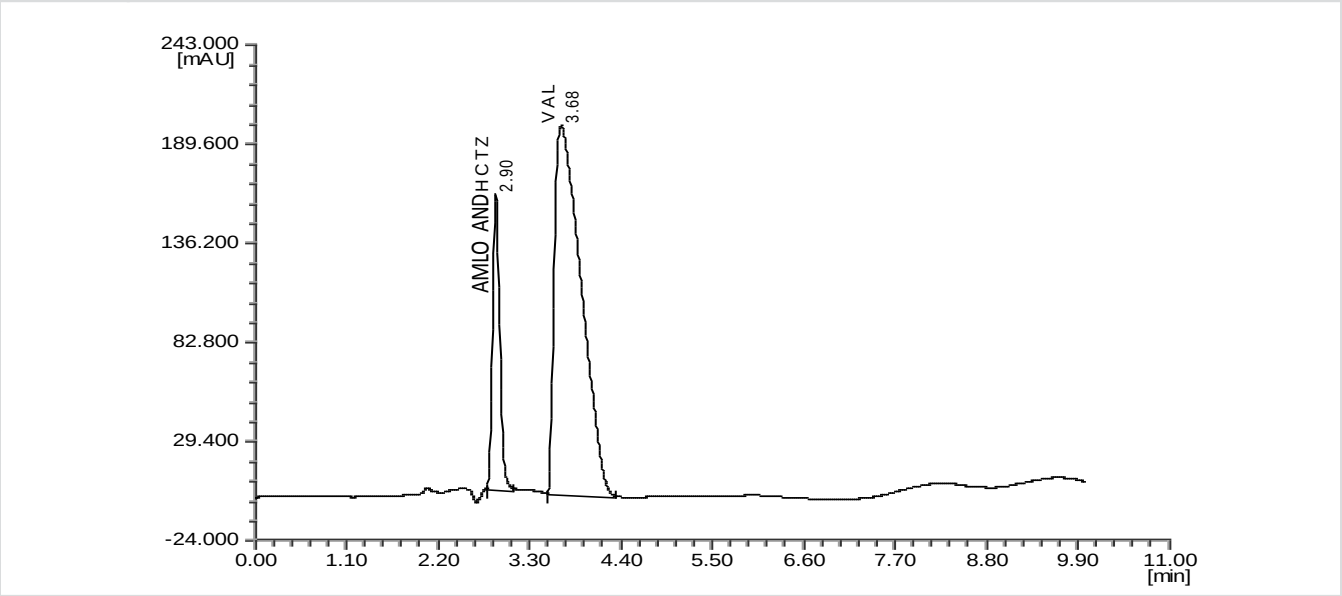


Figure 3.4: Chromatogram showing the effect of a buffered mobile phase on the separation of a mixture of hydrochlorothiazide (HCTZ), valsartan (VAL) and amlodipine (AMLO). Column: Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm). Mobile phase: acetonitrile-0.1 M potassium dihydrogen phosphate pH 4.29-water (60:4:36, % v/v/v). Column temperature: 40 ^{o}C. Flow rate: 1ml/min. Detection: 229 nm. Injection volume: 20 µl.

3.6.3 Effect of different acetonitrile concentrations at pH 2.0

From the previous observation there was no clear resolution using various combinations of mobile phase at pH 4.29. To get a clear indication of how lowering the pH would affect separation of the analyte compounds the pH was adjusted to the extreme acidic conditions that were possible when using the potassium dihydrogen phosphate as buffer. For the next ratios of

inorganic aqueous buffers and acetonitrile tested the pH was adjusted to 2.0 to allow the component that are acidic and those that are basic to separate on basis of their pK_a. To begin with a mobile phase was prepared by mixing acetonitrile-0.1 M KH_2PO_4 pH 2.0-water (60:4:36, % v/v/v). The chromatogram obtained revealed all the peaks for hydrochlorothiazide, valsartan and amlodipine which were clearly resolved.

When a mobile consisting of acetonitrile-0.1 M KH_2PO_4 pH 2.0-water (75:4:21 % v/v/v) was used the chromatogram obtained revealed all the three peaks that of hydrochlorothiazide, valsartan and amlodipine (figure 3.5). The resolution and tailing factors for all the three peaks were within acceptable range (table 3.3). The retention time for the last compound to elute was 6.04 min.

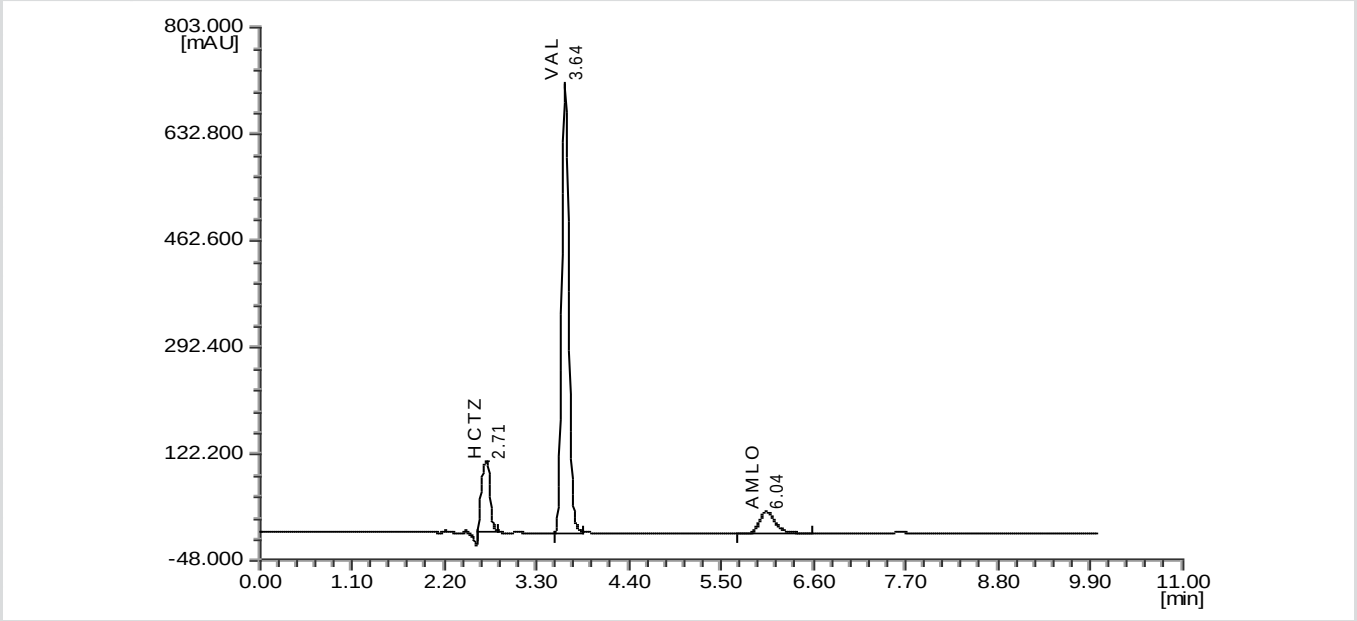


Figure 3.5: Chromatogram showing the effect of acetonitrile concentration on the separation of a mixture of hydrochlorothiazide (HCTZ), valsartan (VAL) and amlodipine (AMLO). Column: Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm). Mobile phase: acetonitrile-0.1 M potassium dihydrogen phosphate pH 2.0-water (75:4:21, % v/v/v). Column temperature: 40 ^{o}C. Flow rate: 1ml/min. Detection: 229 nm. Injection volume: 20 µl.

When a mobile phase consisting of acetonitrile-0.1 M KH_2PO_4 pH 2.0-water (80:4:16, % v/v/v) was used the chromatogram obtained did not have any advantage in peak shape or other

chromatographic parameters. The chromatographic parameters are summarized in figure 3.6 and table 3.3. The capacity factor for amlodipine decreased as the amount of acetonitrile increased while as the capacity factor for the other 2 analytes was unaffected. From the observation made the mobile phase containing acetonitrile-0.1 M KH_2PO_4 pH 2.0-water (75:4:21, % v/v/v) had chromatographic factors within the acceptable limits and symmetrical peak shapes. It was thus chosen for further development.

Table 3.3: Effects of acetonitrile concentration on chromatographic parameters for the separation of amlodipine, valsartan and hydrochlorothiazide

Mobile phase composition Acetonitrile: 0.1 M KH_2PO_4 : water	**Drug**	**Retention time**	**Capacity factor**	**Symmetry factor**	**Resolution**	**Tailing factor**
(60:4:36, %v/v/v)	HCTZ	2.7	2.0	2.0	-	0.8
	VAL	4.4	3.8	1.2	6.7*	1.3
	AMLO	12.1	12.3	1.9	10.0**	1.4
(75:4:21, %v/v/v)						
	HCTZ	2.7	1.7	1.2	-	1.1
	VAL	3.6	3.0	1.8	5.1*	1.3
	AMLO	6.0	5.6	1.5	8.8**	1.7
(80:4:16, %v/v/v)						
	HCTZ	2.7	1.9	1.3	-	1.4
	VAL	3.2	2.6	1.6	2.6*	1.3
	AMLO	5.1	4.6	1.4	8.4**	1.2

Column: Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm). Mobile phase: acetonitrile-0.1 M potassium dihydrogen phosphate pH 2.0-water (75:4:21, % v/v/v). Column temperature: 40 ^{o}C. Flow rate: 1ml/min. Detection: 229 nm. Injection volume: 20 µl. Resolution: *HCTZ/VAL, **VAL/AMLO.

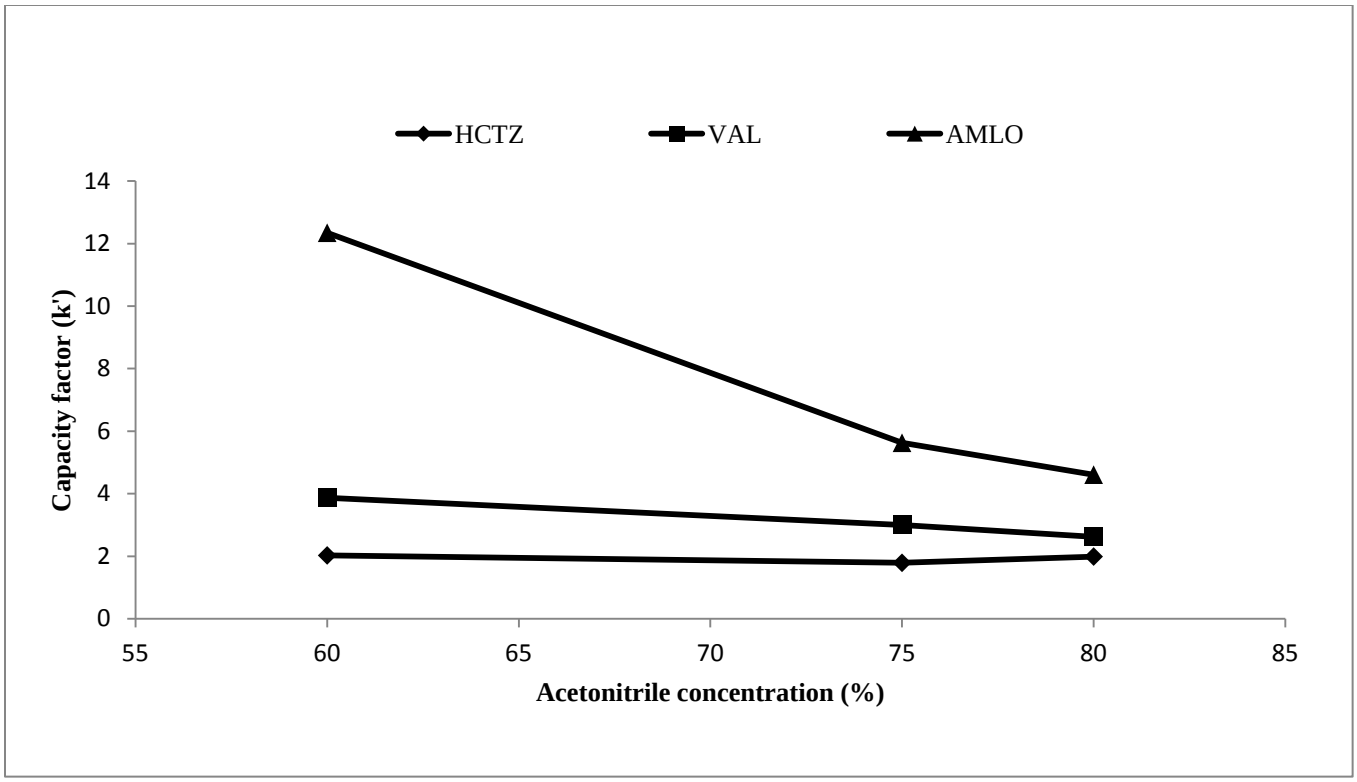


Figure 3.6: Effect of acetonitrile concentration on the capacity factors of hydrochlorothiazide (HCTZ), valsartan (VAL) and amlodipine (AMLO). Column: Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm). Mobile phase: acetonitrile-0.1 M potassium dihydrogen phosphate pH 2.0-water (75:4:21, % v/v/v). Column temperature: 40 ^{o}C. Flow rate: 1ml/min. Detection: 229 nm. Injection volume: 20 µl.

3.6.4 Effect of different concentrations of potassium phosphate buffer

The buffer concentrations were prepared from the stock solution of 0.1M KH_2PO_4 by taking volumes that would give the desired concentration and tested at 0.010, 0.009, 0.008, 0.007, 0.006, 0.005, 0.004 and 0.003 M KH_2PO_4. The mobile phase containing acetonitrile-0.1 M KH_2PO_4-water (75:7:18, % v/v/v) % showed reliable results with all chromatographic parameters within acceptable range. Three injections were done in each case. A similar procedure was followed for all the other buffer strengths and the chromatogram for the optimum buffer concentration is shown below (figure 3.7).

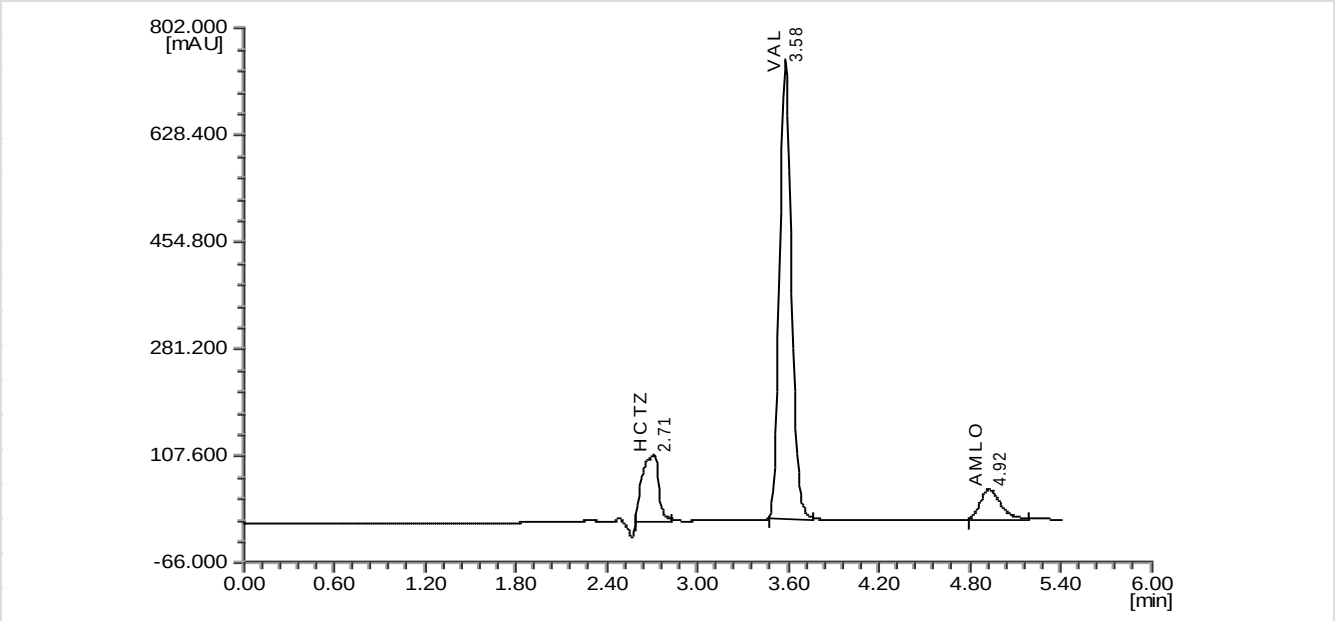


Figure 3.7: Chromatogram showing the effect of buffer concentration on the separation of a mixture of hydrochlorothiazide (HCTZ), valsartan (VAL) and amlodipine (AMLO). Column: Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm). Mobile phase: acetonitrile-0.1 M potassium dihydrogen phosphate pH 2.0-water (75:6:19, % v/v/v). Column temperature: 40 °C. Flow rate: 1ml/min. Detection: 229 nm. Injection volume: 20 µl.

After adjustment of the effective buffer concentration to 0.006 and 0.005 M a slight increase in the retention time of the peak at 4.92 and 5.13 min, respectively, in comparison to the effective concentration of 0.007 M was noted. The peak shape of hydrochlorothiazide was still broad. When an effective buffer concentration of 0.004 M was used there was a slight increase in the retention time of the last to elute at 6.04 in comparison to the peaks of other buffer strengths. The symmetry of hydrochlorothiazide was much better compared to the previous ones.

When an effective buffer concentration of 0.003 M was used there was no advantage in retention time or symmetry of the peaks. Others buffer strengths below and above the ones shown did not have any advantage and their chromatographic parameters were not within range. The buffer with an effective concentration of 0.006 M was selected for further optimization of the method (figure 3.8 and table 3.4).

Table 3.4: Effect of buffer concentration on chromatographic parameters for the separation of amlodipine, valsartan and hydrochlorothiazide

Buffer concentration	Drug	Retention time	Capacity factor	Symmetry factor	Resolution	Tailing factor
0.007M	HCTZ	2.7	1.9	0.8	-	0.91
	VAL	3.5	2.9	1.8	4.6*	1.35
	AMLO	4.6	4.1	1.5	5.2**	1.28
0.006M	HCTZ	2.7	1.9	1.0	-	0.99
	VAL	3.5	2.9	1.6	4.6*	1.23
	AMLO	4.9	4.4	2.0	6.2**	1.54
0.005 M	HCTZ	2.7	1.9	1.3	-	1.14
	VAL	3.5	2.8	1.6	2.6*	1.33
	AMLO	5.1	4.6	1.4	8.4**	1.21
0.004M	HCTZ	2.7	1.9	1.2	-	1.13
	VAL	3.6	2.6	1.8	5.1*	1.39
	AMLO	6.0	5.6	1.5	8.8**	1.74
0.003M	HCTZ	2.7	1.9	0.8	-	0.93
	VAL	3.5	2.9	1.2	4.3*	1.10
	AMLO	6.6	6.3	1.5	10.6**	1.26

Column: Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm). Mobile phase: acetonitrile-0.1 M potassium dihydrogen phosphate pH 2.0-water (75:6:19, % v/v/v). Column temperature: 40 ^{o}C. Flow rate: 1ml/min. Detection: 229 nm. Injection volume: 20 µl. Resolution: *HCTZ/VAL, **VAL/AMLO.

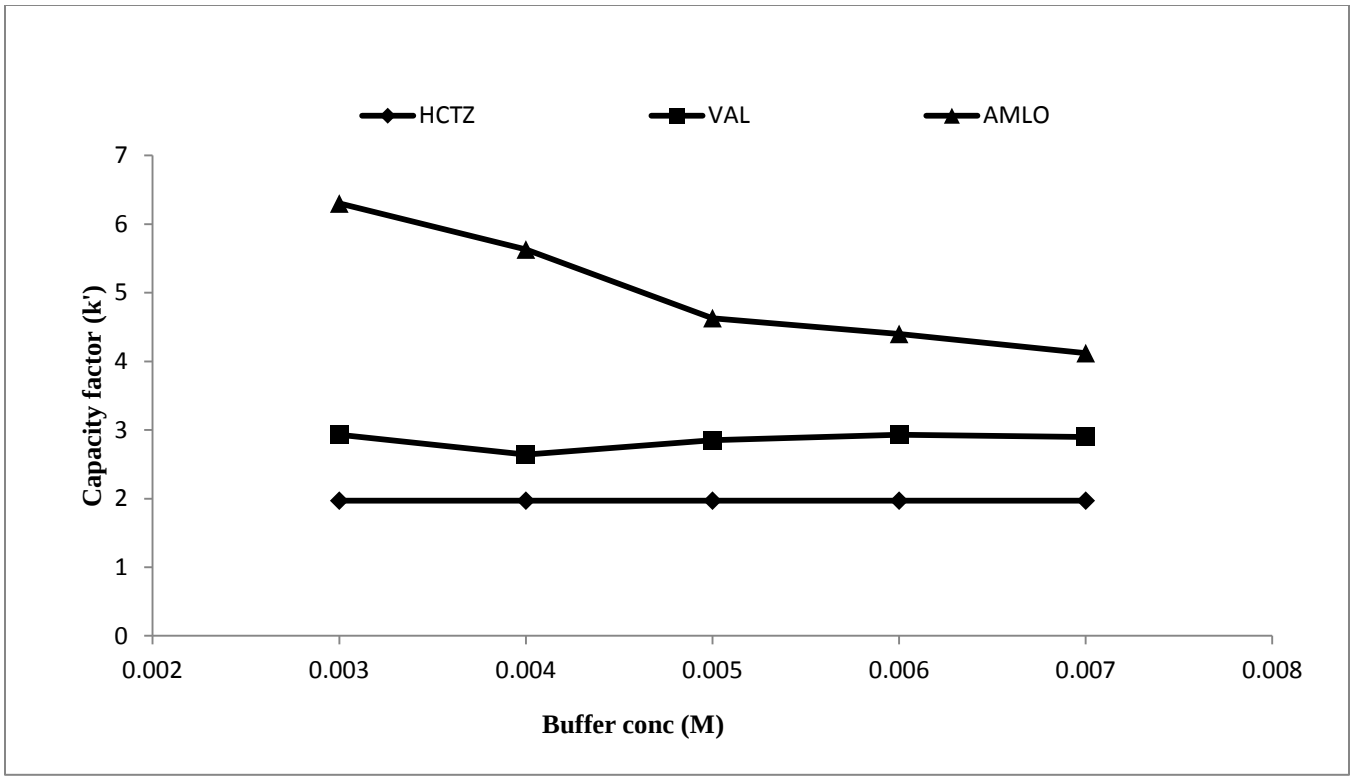


Figure 3.8: Effect of buffer concentration on capacity factors of hydrochlorothiazide (HCTZ), valsartan (VAL) and amlodipine (AMLO). Column: Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm). Mobile phase: acetonitrile-0.1 M potassium dihydrogen phosphate pH 2.0-water (75:6:19, % v/v/v). Column temperature: 40 ^{o}C. Flow rate: 1ml/min. Detection: 229 nm. Injection volume: 20 µl.

3.6.5 Effect of pH

The use of acidic buffers suppresses the ionization of acids. This, in turn, increases hydrophobic interaction, and the resulting faster kinetics yield significantly improved peak shapes in ionic/ionizable species [39]. Hydrochlorothiazide and valsartan are acids while amlodipine is a base. In acidic buffer pH hydrochlorothiazide and valsartan would remain unionized increasing hydrophobic interactions while amlodipine would be ionized increasing hydrophilic interactions thus enhancing separation depending on their pK_a

The effects of adjusting pH of the phosphate buffer on the separation of the components peaks and improving the symmetry factors of the peaks was then investigated. Mobile phases consisting of acetonitrile-0.1 M KH_2PO_4-water (75:6:19, % v/v/v) were prepared, the pH of the buffer was adjusted to 2.0, 2.5 and 3.0 and then used to run the working standard solution.

At pH 2.0 the chromatogram obtained had advantage in resolution and retention time and the run time was within 5 min. The chromatogram obtained with the mobile phase with pH of the buffer adjusted to 2.5 showed good separations of the peak components and had good resolution and peak symmetry but the peak shape for hydrochlorothiazide was not satisfactory. The retention time for amlodipine the last to elute was 4.95 min.

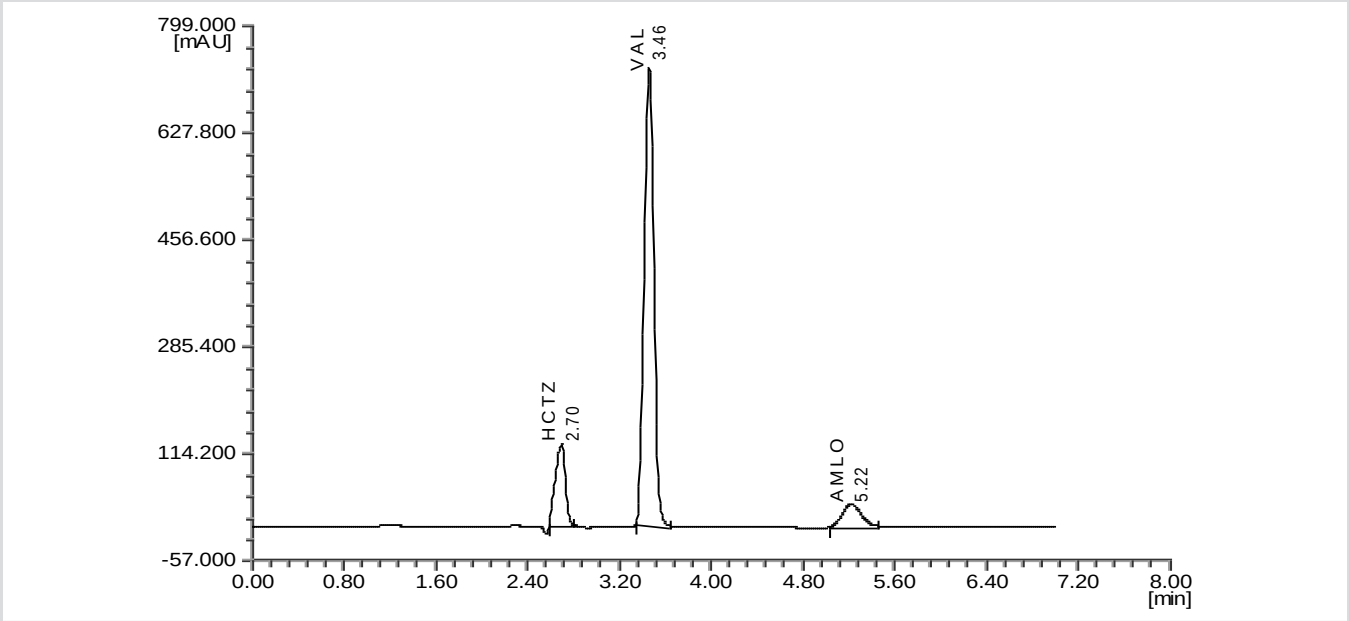


Figure 3.9: Chromatogram showing the effect of pH on the separation of a mixture of hydrochlorothiazide (HCTZ), valsartan (VAL) and amlodipine (AMLO). Column: Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm). Mobile phase: acetonitrile-0.1 M potassium dihydrogen phosphate pH 3.0-water (75:6:19, % v/v/v). Column temperature: 40 ^{o}C. Flow rate: 1ml/min. Detection: 229 nm. Injection volume: 20 µl.

The chromatogram obtained with the mobile phase with pH of the buffer adjusted to 3.0 showed the best separation of the peak components (figure 3.9) had good resolution and peak symmetry. The retention time for amlodipine the last to elute was 5.22 min. Other pH values above 3.0 were tested but did not have any advantage in resolution or peak symmetry.

The effect of pH on the retention, resolution and symmetry of different components peaks are summarized below (table 3.5 and figure 3.10). The optimum pH for the separation of the components peaks was 3.0 and it was used for further investigation.

Table 3.5: Effect of pH on the chromatographic parameters for the separation of amlodipine, hydrochlorothiazide and valsartan

pH	Drug	Retention Time	Capacity factor	Symmetry factor	Resolution	Tailing factor
2.0	HCTZ	2.7	1.9	1.0	-	0.9
	VAL	3.5	2.9	1.6	4.6*	1.2
	AMLO	4.9	4.4	2.0	6.2**	1.5
2.5	HCTZ	2.6	1.9	1.0	-	1.0
	VAL	3.5	2.9	1.1	5.0*	1.3
	AMLO	4.9	4.0	1.1	6.5**	1.2
3.0	HCTZ	2.7	1.9	1.0	-	0.9
	VAL	3.4	2.8	1.5	4.4*	1.1
	AMLO	5.2	4.7	1.3	6.7**	1.1

Column: Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm). Mobile phase: acetonitrile-0.1 M potassium dihydrogen phosphate pH 3.0-water (75:6:19, % v/v/v). Column temperature: 40 ^{o}C. Flow rate: 1ml/min. Detection: 229 nm. Injection volume: 20 µl. Resolution: *HCTZ/VAL, **VAL/AMLO.

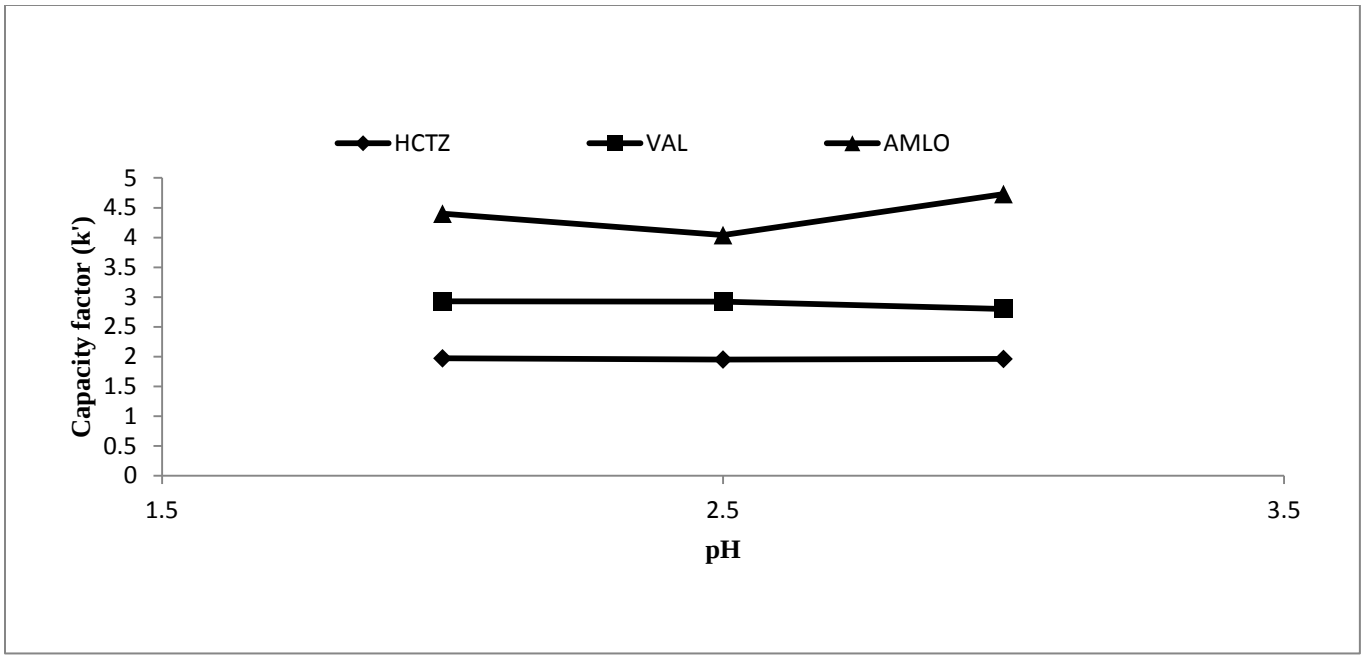


Figure 3.10: Effects of pH on capacity factor for the separation of a mixture of hydrochlorothiazide (HCTZ), valsartan (VAL) and amlodipine (AMLO). Column: Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm). Mobile phase: acetonitrile-0.1 M potassium dihydrogen phosphate pH 2.0-3.0-water (75:6:19, % v/v/v). Column temperature: 40 ^{o}C. Flow rate: 1ml/min. Detection: 229 nm. Injection volume: 20 µl

3.6.6 Effect of temperature

In conventional reversed-phase separations, raising the temperature is usually the preferable option, as this decreases retention time as well as back pressure. The latter effect makes it possible to use small stationary phase particles particle sizes (3-1.7) µm. Improved efficiency (higher number of theoretical plates) is achieved not only by using small particles, but additionally lowering the viscosity of the eluent, thus increasing kinetics. Accordingly, at a flow rate of 2 ml/min at 80 ^{o}C in four 10 cm columns in serial arrangement, the retention time is equal to that of a 25 cm column at 30 ^{o}C and a flow rate of 1 ml/min, but efficiency is increased by a factor of 4. As the increase in temperature leads to a decrease of polarity in the eluent, fewer organic components are needed in the eluent [39].

The effect of different column temperatures on the separation of the components peaks and improving the symmetry factors of the observed signals were then investigated. The temperatures investigated were 25-50 ^{o}C at 5^{o} intervals. Mobile phases consisting of acetonitrile-0.1 M KH_2PO_4 pH 3.0-water (75:6:19, % v/v/v), were prepared. At 25 ^{o}C the chromatogram obtained showed good separation of the components peak. The retention time for amlodipine the last to elute was 5.39 min. The symmetry of amlodipine and valsartan was within range but the peak of hydrochlorothiazide had a shoulder. The chromatogram obtained with the temperature maintained at 30 ^{o}C showed good separation of the components peaks. Peak symmetry was better and showed advantage in retention time. The retention time for amlodipine the last to elute was 5.16 min compared to 5.39 min for the column temperature was maintained at 25 ^{o}C. The chromatographic parameters of all peaks were within acceptable range (table 3.6). The chromatogram obtained with the temperature maintained at 35 ^{o}C showed good separation of the components peaks. Peak shapes were better and showed advantage in retention time. The Column was thereafter maintained at 40 ^{o}C and the process of injection repeated. The results are shown in figure 3.11 below.

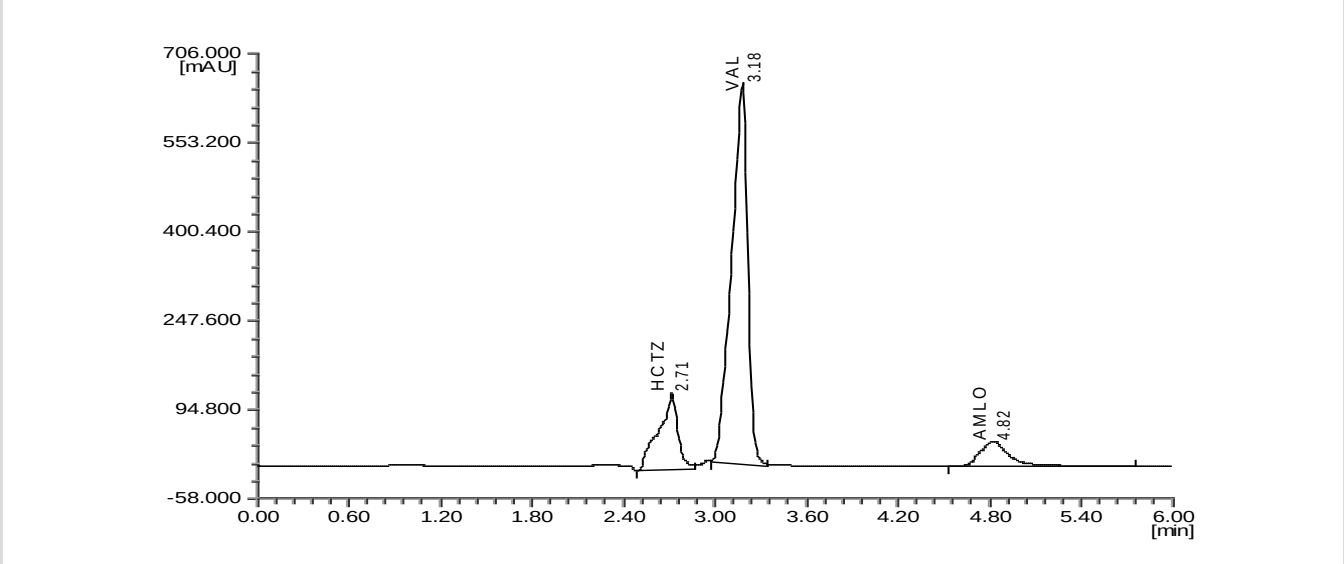


Figure 3.11: Chromatogram showing the effect of temperature on the separation of a mixture of hydrochlorothiazide (HCTZ), valsartan (VAL) and amlodipine (AMLO). Column: Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm). Mobile phase: acetonitrile-0.1 M potassium dihydrogen phosphate pH 3.0-water (75:6:19, % v/v/v). Column temperature: 40 ^{o}C. Flow rate: 1ml/min. Detection: 229 nm. Injection volume: 20 µl.

The chromatogram obtained with the temperature maintained at 40 ^{o}C showed good separation of the components peak. Peak symmetry and shapes were better and showed advantage in retention time of the last component to elute. The chromatogram obtained with the temperature maintained at 45 ^{o}C did not show good separation of the components peak. The retention time for amlodipine, the last to elute, was 4.68 min compared to 4.82 min for the column maintained at 40 ^{o}C. The chromatogram obtained with the temperature maintained at 50 ^{o}C did not show good separation of the components peak. The retention time for amlodipine, the last to elute, was 4.54 min compared to 4.68 min for the column maintained at 45 ^{o}C.

Table 3.6: Effect of temperature on chromatographic parameters for the separation of amlodipine, valsartan and hydrochlorothiazide

Temperature (^{O}C)	Drug	Retention Time	Capacity factor	Resolution	Tailing Factor	Symmetry Factor
	HCTZ	2.7	2.0	-	0.9	0.9
25	VAL	3.3	2.7	2.0*	0.9	0.7
	AMLO	5.3	4.9	5.4**	1.1	1.3
	HCTZ	2.7	2.0	-	0.9	0.8
30	VAL	3.2	2.5	2.1*	0.9	1.1
	AMLO	5.1	4.6	6.0**	1.2	1.5
.	HCTZ	2.3	2.0	-	0.9	0.9
35	VAL	3.2	2.5	2.0*	0.8	0.8
	AMLO	5.1	4.5	5.7**	1.1	1.2
	HCTZ	2.7	1.9	-	0.8	0.6
40	VAL	3.1	2.4	2.0*	0.8	0.8
	AMLO	4.8	4.2	6.1**	2.2	1.1
	HCTZ	2.6	1.9	-	0.9	0.8
45	VAL	3.1	2.4	1.8	0.7	0.6
	AMLO	4.6	4.1	5.8**	1.1	1.2
	HCTZ	2.6	1.9	-	0.9	0.8
50	VAL	3.0	2.3	1.7*	0.8	0.8
	AMLO	4.5	3.9	5.7**	1.7	1.7

Column: Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm). Mobile phase: acetonitrile-0.1 M potassium dihydrogen phosphate pH 3.0-water (75:6:19, % v/v/v) respectively: Column temperature: 40 ^{o}C. Flow rate: 1ml/min. Detection: 229 nm. Injection volume: 20 µl. Resolution: *HCTZ/VAL, **VAL/AMLO.

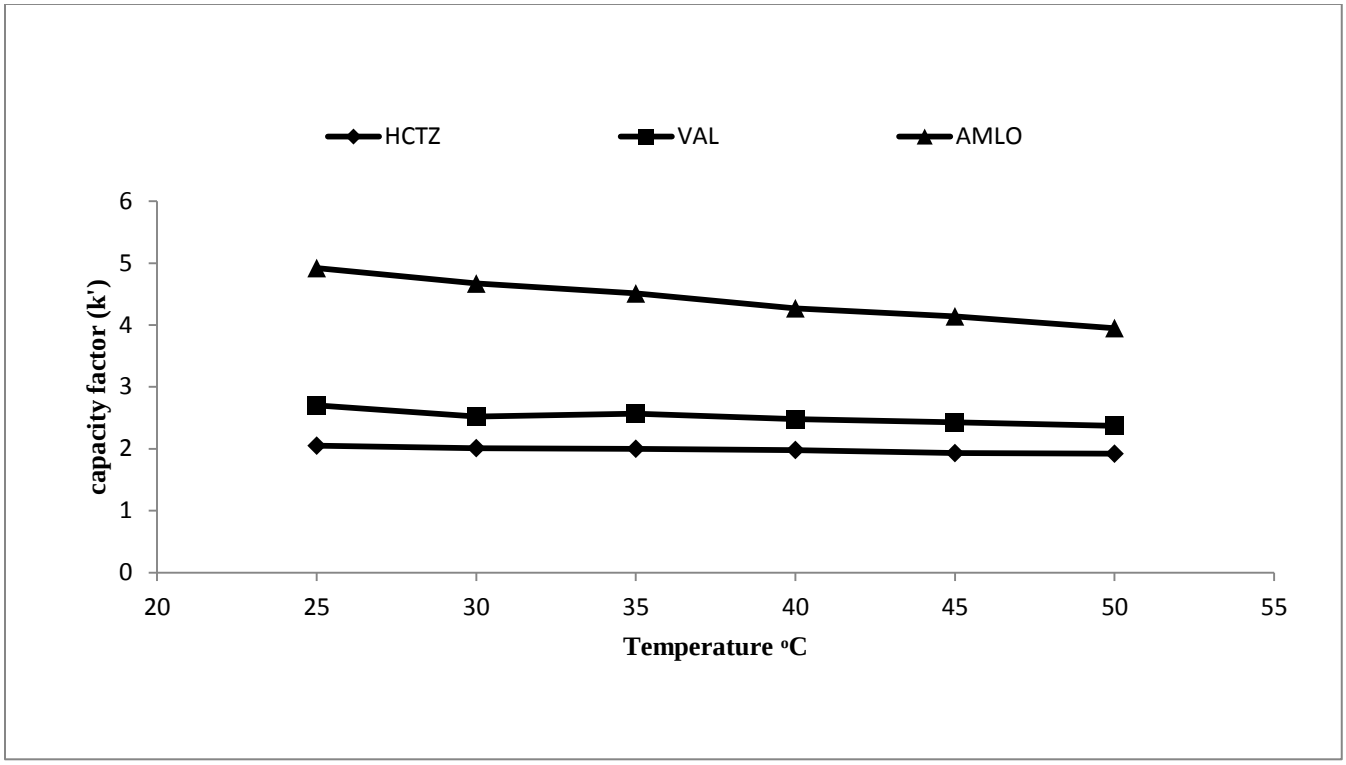


Figure 3.12: Effect of temperature on capacity factors of hydrochlorothiazide (HCTZ), valsartan (VAL) and amlodipine (AMLO). Column: Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm). Mobile phase: acetonitrile-0.1 M potassium dihydrogen phosphate pH 3.0-water (75:6:19, % v/v/v). Column temperature: 25-50 °C. Flow rate: 1ml/min. Detection: 229 nm. Injection volume: 20 µl.

The effect of temperature on the retention, resolution and symmetry of different components peaks are summarized in table 3.6 and figure 3.12. Generally temperature did not affect much the capacity factor of hydrochlorothiazide and valsartan but decreased with increase in temperature for amlodipine. Column temperature maintained at 40 °C gave the best separation and was thus selected as the optimum.

3.6.7 Effect of sample solvent

In the method development individual working standards were dissolved in acetonitrile. From the stock solution, the mixed working solution was prepared by pipetting 1 ml of each of the stock solutions into the same 25 ml volumetric flask and made up to the volume with acetonitrile: water (50:50, % v/v). At this point, the mixed working solution was prepared by pipetting 1 ml of each of the stock solutions into the same 25 ml volumetric flask and making up

to the volume with acetonitrile: 0.1 M KH_2PO_4 pH 3: water (75:6:19, % v/v/v). When this solution was injected the peaks obtained were narrow, resolved and symmetrical. The parameters improved and peak splitting observed with hydrochlorothiazide was no longer evident (figure 3.13 and table 3.7).

It was evident that use of acetonitrile: water (50:50, %v/v) as the solvent for working standards instead of the mobile phase was contributing to the poor peak symmetry and resolution. Sample solutions were there after prepared using the mobile phase.

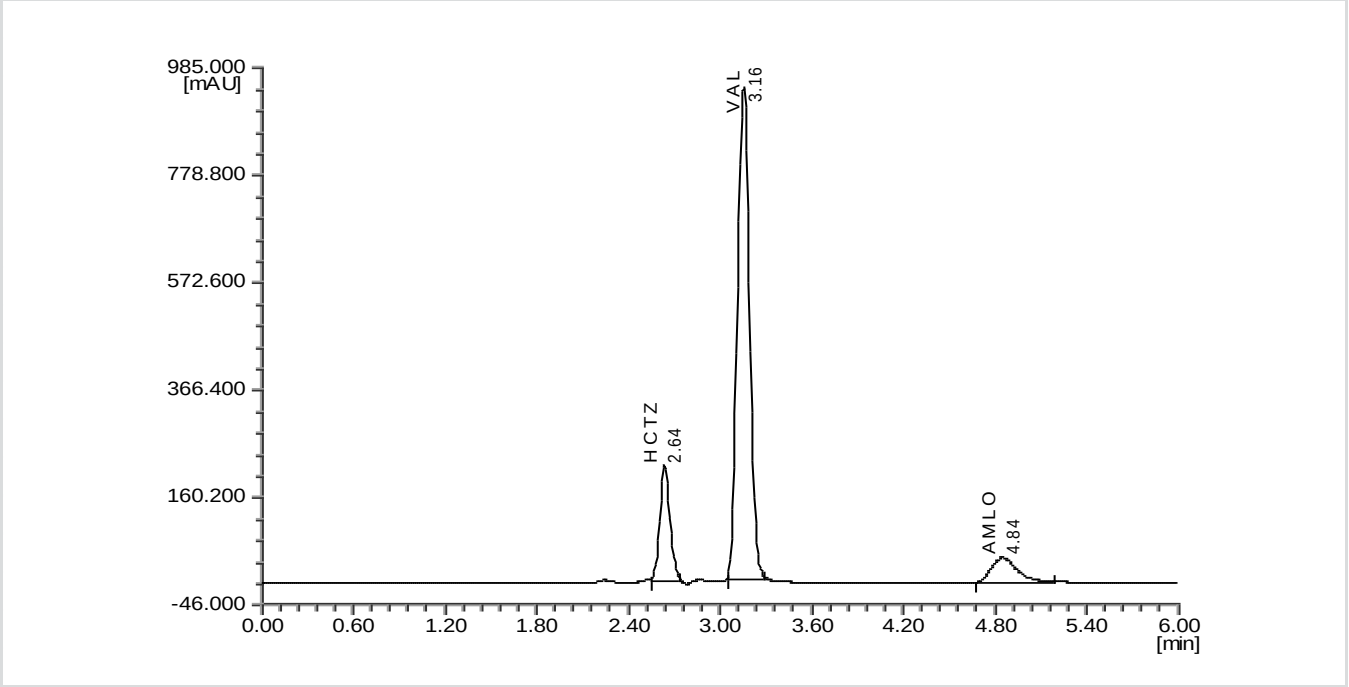


Figure 3.13: Chromatogram showing the effect of sample solvent on the separation of a mixture of hydrochlorothiazide (HCTZ), valsartan (VAL) and amlodipine (AMLO). Column: Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm). Mobile phase: acetonitrile-0.1 M potassium dihydrogen phosphate pH 3.0-water (75:6:19, % v/v/v). Column temperature: 40 ^{o}C. Flow rate: 1ml/min. Detection: 229 nm. Injection volume: 20 µl.

Table 3.7: Effect of sample solvent on chromatographic parameters for the separation of hydrochlorothiazide, valsartan and amlodipine

Drug	Retention time	k'	Resolution	Tailing factor	Symmetry factor
HCTZ	2.6	1.8	-	1.1	1.2
VAL	3.1	2.4	3.6*	1.1	1.2
AMLO	4.8	4.3	6.9**	1.4	1.9

Column: Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm). Mobile phase: acetonitrile-0.1 M potassium dihydrogen phosphate pH 3.0-water (75:6:19, % v/v/v). Column temperature: 40 ^{o}C. Flow rate: 1ml/min. Detection: 229 nm. Injection volume: 20 µl. Resolution: *HCTZ/VAL, **VAL/AMLO.

An acidic compound will ionize in a basic medium while a base will ionize in an acidic medium. The predominant compound will depend on the pK_a or pK_b and the pH. The lower the pK_a the higher the degree of ionization and the stronger the acid is. The unionized compound will partition itself in the organic solvent while the ionized will partition in the aqueous solvent. When hydrochlorothiazide and valsartan are dissolved in the mobile phase which is at acidic pH they remain largely unionized and will partition on the organic phase while amlodipine which is basic will be ionized and would partition in the aqueous phase. Therefore matching the contents and conditions of the mobile phase and sample solvent ensured that the dissociation equilibrium was maintained and this enhanced selectivity there by improving peak symmetry and resolution. Based on this it is expected that amlodipine would be the first to elute but from the chromatogram amlodipine is the last to elute. Interactions between the ionized amlodipine with the remnant silanols of the stationary phase through ionic and hydrogen bonds might explain this observation.

3.6.8 Optimum chromatographic conditions

The optimum conditions for the separation of the amlodipine, valsartan and hydrochlorothiazide were Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm) column and a mobile phase consisting of acetonitrile-0.1 M KH_2PO_4 pH 3.0-water (75:6:19, % v/v/v). Column temperature was

maintained at 40 ^{o}C and the flow rate was 1ml/min: Detection was at 229 nm and injection of 20 µl (figure 3.14).

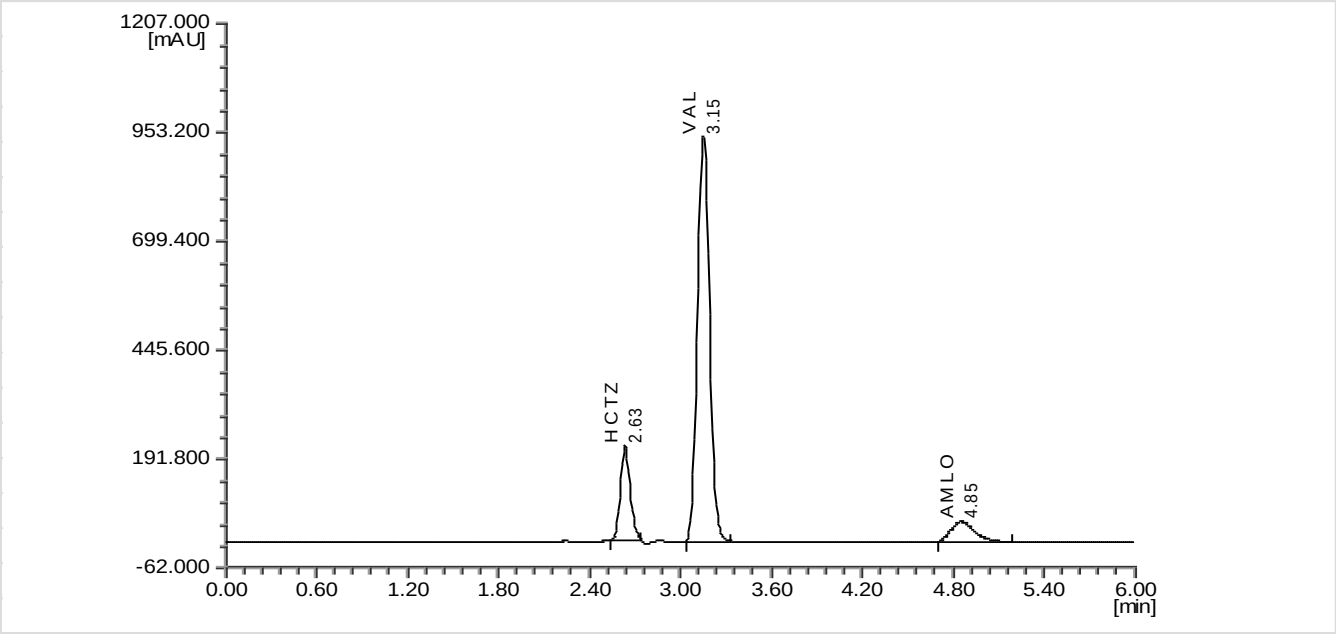


Figure 3.14: Chromatogram for the separation of a mixture of hydrochlorothiazide (HCTZ), valsartan (VAL) and amlodipine (AMLO) at optimal conditions. Column: Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm). Mobile phase: acetonitrile-0.1 M potassium dihydrogen phosphate pH 3.0-water (75:6:19, % v/v/v). Column temperature: 40 ^{o}C. Flow rate: 1ml/min. Detection: 229 nm. Injection volume: 20 µl.

CHAPTER FOUR

METHOD VALIDATION

4.1 Introduction

Method validation is the process of determining, through laboratory studies, that the performance characteristics of the method meet the requirements for its intended analytical applications. The characteristics that are studied during method validation include sensitivity, accuracy, precision, specificity, linearity of detector response, range of analyte concentration and robustness. Accuracy of a method is usually determined by comparing the assay results obtained using the method under validation against those obtained using an established and well validated pre-existing method. In the case of the test method under development, the accuracy for the developed method is determined by spiking the finished commercial products with working standards of compounds under study. The analytical characteristics used in this method validation were accuracy, precision, linearity, limit of detection, limit of quantification and robustness [40-41].

4.2 Accuracy

The accuracy of an analytical method is the closeness of the test results obtained by that procedure to a true known value. The accuracy for the developed method was determined by spiking the finished commercial products with working standards of compounds under study. The difference between spiked sample result and the unspiked sample resulted was calculated as percentage of the known added spike concentration. For this purpose one commercial compound that contained all the 3 compounds was used. The standards were added to the samples at 3 concentrations, 80, 100 and 120% of the assay concentrations and injected in triplicate. The standards were added at amounts that would increase the concentration by 20%. The % recovery of each added working standard was regarded as the accuracy (table 4.1).

Table 4.1: Percentage recovery of hydrochlorothiazide, valsartan and amlodipine

Drug	% recovery	Coefficient of variance (CV) n = 9
Hydrochlorothiazide	100.6	1.82
Valsartan	99.9	0.45
Amlodipine	99.7	1.24

The recoveries of all compounds were within the specified guidelines (ICH) of 98-103% [40-41].

4.3 Precision

Precision is expressed as the coefficient of variation of the results obtained from analysis of a series of repeated determinations of a sample using the method being evaluated. Three levels of precision namely repeatability, intermediate precision and reproducibility are assessed. The coefficient of variation of six replicate injections run on the same day is used as a measure of repeatability. Intermediate precision is determined using the coefficient of variation of various assays done on different days. This study focused on inter-day variability as a measure of intermediate precision. Reproducibility is assessed by means of inter-laboratory trials but this was not done during the course of this study.

The intraday variation was determined by preparing three working standard solutions containing 10 µg/ml, 80 µg/ml and 20 µg/ml, of hydrochlorothiazide, valsartan and amlodipine respectively on the same day and subsequently injecting each solution into the chromatography system six times. The coefficient of variation of the peak areas was calculated.

The inter-day precision of the method was determined by running six replicate injections of a freshly prepared standard solution after every three day interval. Fresh mobile phase was prepared for each day of analysis. The component peak areas obtained were all normalized to the

desired concentration and the coefficient of variation of normalized areas calculated to determine the inter-day precision of the method. The ICH guidelines recommend that n should be at least 6 and 36 for repeatability and intermediate precision respectively. The results obtained are summarized in table 4.2 [41, 42]. The coefficient of variation in both cases was below 2% indicating that the method was precise.

Table 4.2: Repeatability and intermediate precision for hydrochlorothiazide, valsartan and amlodipine

Drug	Repeatability peak areas	Intermediate precision
	Coefficient of variation (n=18)	Coefficient of variation (n=36)
Hydrochlorothiazide	0.629	1.080
Valsartan	0.298	0.435
Amlodipine	0.724	1.412

4.4 Linearity of detector response

The linearity of detector response is its ability to obtain test results that are directly proportional to the concentration of the analyte in the sample within a given range. The range of an analytical procedure is the interval between the upper and the lower concentration of analyte in the sample for which it has been demonstrated to have a suitable level of linearity, precision and accuracy.

The linearity of the method was determined by preparing a standard stock solution from which working solutions were prepared by diluting appropriately to yield solutions containing 25, 50, 75, 100, 125 and 150% of the working standard solution concentration. Each of these solutions was then analyzed in triplicate and the peak areas obtained for each analyte compound plotted against concentration.

The data obtained from the linearity determination experiments was subjected to linear regression analysis. The linearity equations were $y = 4537\ x + 26628$, $R^2 = 0.991$ for hydrochlorothiazide, $y = 5344\ x + 124106$, $R^2 = 0.997$ for valsartan and $y = 4227x + 9893$, $R^2 = 0.995$ for amlodipine. Correlation coefficient R^2 values were in all cases greater than 0.99

indicating a strong correlation between the concentrations of the analytes and the peak areas and therefore the method could be applied in the assay of all or any of the three analyte compounds. A summary of the linearity analysis results obtained is shown in table 4.3. The ICH guidelines recommend that for the establishment of linearity a minimum of five concentrations be utilized over the range of 80 to 120 % [41, 42]. This method was found to be linear over the range tested for all the three compounds.

Table 4.3: Linear regression analysis for hydrochlorothiazide, valsartan and amlodipine

Drug	Slope	Y intercept	R^2 value
Hydrochlorothiazide	4537	26628	0.991
Valsartan	5344	124106	0.997
Amlodipine	4227	9893	0.995

4.5 Sensitivity

Limit of detection

The limit of detection (LOD) is the lowest amount of analyte in a sample that can be detected but not necessarily quantified using an analytical technique under specified experimental conditions. The LOD was established by signal over noise (S/N) method where 6 injection of blank (mobile phase) were injected and the mean peak height was determined. A serial dilution of a solution containing 10 g/ml, 40 g/mland 5 g/ml of hydrochlorothiazide, valsartan and amlodipine respectively was prepared and the concentration that gave signal to noise ratio of 3 considered to be the LOD and expressed as nano grams (ng). The signal to noise ratio values of the component peaks were determined with reference to a blank injection of the diluents solution run under the same liquid chromatographic conditions [41, 42]. The results obtained are summarized in table 4.4 below.

Limit of quantitation

The limit of quantitation (LOQ) is the lowest amount of analyte in a sample that can be determined with acceptable precision under specified experimental conditions. The degree of

precision considered to be acceptable for purposes of LOQ determination from peak areas of six injections (n = 6) is CV (coefficient of variance) less than 20% and at a signal to noise ratio of 10:1 [40, 41].

The concentration that gave signal to noise ratio of 10 of the serial dilution of the solution made in determination of limit of detection was considered to be the LOQ and expressed as nanograms (ng) (table 4.6. below)

Table 4.4: Limit of detection and limit of quantitation for hydrochlorothiazide, valsartan and amlodipine

Drug	LOD (ng)	LOQ (ng)	Peak areas CV at LOQ
Hydrochlorothiazide	10.72	35.76	1.23
Valsartan	21.20	71.23	1.55
Amlodipine	14.45	48.16	1.76

Determination of LOD is indicated for method developed for determination of restricted substances and degradation products that are usually present in small quantities in the sample [42] while the LOQ was determined for this method to give an idea of the amount of each compound which can be quantified with adequate precision and accuracy to enable the profiling of related substances and degradation products during stability studies. The low limit of detection indicates that the developed method can be used for detection of low concentration of active constituents extracted from body fluids and tissues.

4.6 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage [42]. In liquid chromatography variations of pH in mobile phase, mobile phase composition, column temperature and flow rate are considered.

In this study the effect of making small but deliberate adjustments in the optimized chromatographic factors was investigated. The factors adjusted were pH of the buffer, column temperature and mobile phase composition. Robustness of the method was determined from the degree of variation observed in peak areas and retention times from the same working standard solution analyzed while adjusting each of the liquid chromatographic factors indicated. Six replicate injections of the same working standard solution were run after having adjusted a single chromatographic parameter and coefficient of variation of peak areas of component peaks calculated. The degree of variation observed was then used to infer the method's robustness.

The influence of changing each of the three chromatographic factors was tested at 3 levels. The buffer pH was studied at 2.75, 3.0 and 3.25; column temperature at 35, 40 and 45 ^{o}C while influence of acetonitrile concentration was tested at 70, 75 and 80%. At the ranges which pH was tested hydrochlorothiazide and valsartan remained largely unaffected while the capacity factor of amlodipine decreased slightly. This is because amlodipine is ionized at pH 3.0. The mobile phase composition caused a gradual decrease in capacity factor for amlodipine but hydrochlorothiazide and valsartan were not affected. Temperature also had some decreasing effect on the capacity factor of amlodipine with the other two remaining unaffected.

The influence of changing each of the three chromatographic factors had the greatest impact on amlodipine, while hydrochlorothiazide and valsartan remained largely unaffected. From the observation made there is need for accurate determination of pH to minimize deviations from optimum during the application of the method (table 4.5 and appendix 7).

Changing each of the three chromatographic factors had no effect on the peak areas and the coefficient of variance in all cases was below 2%. The resolution of all the compounds remained unaffected by variation of these factors. Generally the method was robust within the ranges of the critical factors investigated.

Table 4.5: Summary of robustness of the method for hydrochlorothiazide, valsartan and amlodipine

		CV
Factors altered	**Compound**	

		Peak area
Column temperature (35, 40, 45) ˚C	Hydrochlorothiazide	0.400
	Valsartan	0.506
	Amlodipine	1.697
Mobile phase buffer pH (2, 2.5, 3)	Hydrochlorothiazide	0.374
	Valsartan	1.163
	Amlodipine	1.556
Mobile phase acetonitrile concentration (75, 80, 85% v/v)	Hydrochlorothiazide	1.593
	Valsartan	0.651
	Amlodipine	1.461

CHAPTER FIVE

ANALYSIS OF COMMERCIAL SAMPLES

5.1 Introduction

Analytical methods development and validation play important roles in the discovery, development, and manufacture of pharmaceuticals. The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose such as active pharmaceutical ingredients and drug product analysis and stability indicating methods. This validation has to be in accordance with ICH guidance documents and current Good Manufacturing Practices.

The objective of this analytical method developed and validated was to create a reliable technique that may be employed in the analysis of commercial samples and was intended for use in evaluating the quality of antihypertensive drugs containing any or all three compounds of interest as active ingredients. Most antihypertensive drugs in the Kenyan market contain either one, two or all three components under study. The method developed can be used to analyze these three categories. There is currently only one fixed dose combination in the Kenyan market that contains all the three compounds but more products can be expected in the Kenyan market soon.

5.2 Acquisition of commercial samples

The test samples were purchased from randomly selected retail pharmacies located within the Thika town. A total of 6 commercial samples were obtained. For each of the sample, its batch number, manufacturing date, expiry date and the label claim were recorded as in table 5.1 and coded A-E. In each sample three batches were assayed.

Table 5.1: Label details of analyzed samples

Product code	Batch number	Date of manufacture	Expiry date	Label claim
A	S0157	11/2011	10/2013	Amlodipine 5mg Valsartan 160mg Hydrochlorothiazide 12.5mg
	S0100B	11/2011	10/2013	Amlodipine 10mg Valsartan 160mg Hydrochlorothiazide12.5mg
	S0182	02/ 2012	10/ 2014	Amlodipine 10mg Valsartan 160mg Hydrochlorothiazide12.5mg
B	B8136	01/2012	12/ 2013	Amlodipine 10mg Valsartan 160mg
	S0260	09/2011	08/ 2013	Amlodipine 10mg Valsartan 160mg
	B5208	03/2012	02/ 2014	Amlodipine 5 mg Valsartan 160mg
C	T6017	03/ 2011	04/2014	Valsartan 80mg Hydrochlorothiazide 12.5mg
	T3287	03/2012	02/ 2015	Valsartan 80mg Hydrochlorothiazide 12.5mg
	T4530	05/2012	02/2016	Valsartan 80mg Hydrochlorothiazide 12.5mg
	D8258	01/ 2012	12/ 2014	Valsartan 80mg

D	B8067	03/ 2011	02/ 2014	Valsartan 80mg
	D8124	07/2011	06/ 2014	Valsartan 80mg
E	ACTP0041	01/2011	06/ 2013	Amlodipine 10mg
	SKK2300	01/2012	12/ 2013	Amlodipine 5mg
	EII276	01 2012	12/ 2013	Amlodipine 5mg
F	54562	03/ 2011	02/2014	Hydrochlorothiazide 50mg
	20725	07/ 2012	06/ 2016	Hydrochlorothiazide 50mg
	30422	03/ 2012	02/ 2016	Hydrochlorothiazide 50mg

5.3. Analysis of commercial samples

The above batches of each product were sampled for testing and analyzed using the developed HPLC method. The test and standard solutions were run in triplicates for each solution. For each sample, 20 tablets were taken in each case, crushed and powders equivalent to required weight were weighed and dissolved in acetonitrile in 50 ml volumetric flasks. One ml of each of this was further diluted with the mobile phase to make the desired concentration.

The samples were prepared so as to contain 80 µg/ml of valsartan in cases where it was co-formulated with the other two and the concentration of the other two or either of the two was calculated. In cases where individual drugs were present in tablets concentration were made so as to contain 10 µg/ml of amlodipine, 80 µg/ml of valsartan and 20 µg/ml of hydrochlorothiazide, so as to compare with the concentrations of standard solutions. A working standard solution with equivalent concentrations was also prepared.

Although no monograph for the sample combinations are present in official pharmacopoeia, the assay limits specified in the British Pharmacopoeia and United States Pharmacopoeia for single component containing any of the three ingredients were used as a basis for determining whether the products met quality specifications. In all the three cases, the pharmacopoeia specified assay limits of 90-110% for each drug component [12, 14] (table 5.2).

Table 5.2: Analyzed content of hydrochlorothiazide, valsartan and amlodipine in commercial samples

Product code	Batch number	HCTZ % Label claim (CV)	VAL %Label claim (CV)	AMLO % Label claim (CV)
	S0157	81.3 (0.82)	84.3 (0.94)	123.7 (1.25)
A	S0100B	100.3 (1.65)	100.5 (1.61)	99.3 (0.55)
	S0182	89.1 (1.72)	96.5 (1.94)	95.6 (0.75)
	B8136	_	88.9 (1.82)	122.4 (1.69)
B	S0260	_	88.0 (1.87)	122.7 (1.45)
	B5208	_	92.8 (1.28)	129.2 (1.32)
	T6017	74.7 (1.85)	79.9 (1.10)	_
C	T3287	80.6 (1.55)	79.6 (1.47)	_
	T4530	77.3 (1.59)	89.7 (0.41)	_
	D8258	_	79.9 (1.11)	_
D	B8067	_	100.1 (1.23)	_
	D8124	_	77.4 (0.13)	_
	ACTP0041	_	_	123.4 (1.58)
E	SKK2300	_	_	127.6 (1.40)
	EII276	_	_	129.6 (1.21)
F	54562	73.2 (1.17)	_	_
	20725	71.4 (1.11)	_	_
	30422	79.6 (1.92)	_	_

The most noticeable feature was the high content in most of samples of the amlodipine component whose assay value was found to be more than 110% with content as high as 129.6% thus failing to comply in seven batches out of nine with the assay limits defined in the B.P. and U.S.P. Hydrochlorothiazide in eight cases out of nine had low content than the specified in BP and USP. Valsartan complied in four batches from the twelve batches analyzed.

Assay results indicated that there was no significant inter-batch variation in the content of active ingredients in the products tested except in batches under product code A and D. Possible reasons for inter-batch variability may be due to poor manufacturing practices or instability of the active ingredients. Methodological variability of sample solution preparations were ruled out as a possible cause of inter-batch and inter-product variation because validation procedures showed there was good recovery. A typical chromatogram of a sample containing the three, amlodipine valsartan and hydrochlorothiazide is shown below (figure 5.1).

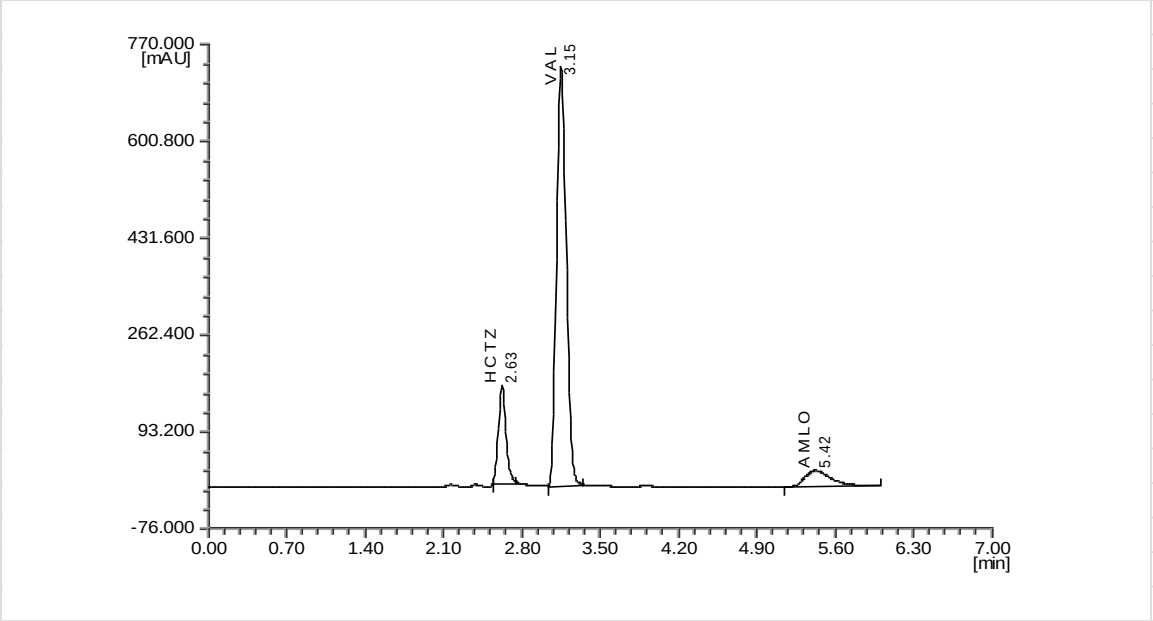


Figure 5.1: Typical chromatogram for the assay of commercial sample containing hydrochlorothiazide (HCTZ), valsartan (VAL) and amlodipine (AMLO). Column: Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm). Mobile phase: acetonitrile-0.1 M potassium dihydrogen phosphate pH 3.0-water (75:6:19, % v/v/v). Column temperature: 40 ^{o}C. Flow rate: 1ml/min. Detection: 229 nm. Injection volume: 20 µl.

CHAPTER SIX

DISCUSSION, CONCLUSION & RECOMMENDATIONS

6.1 Discussion

In this study a method for the simultaneous determination of hydrochlorothiazide, valsartan and amlodipine an antihypertensive fixed dose combination in the market was developed. The optimum conditions for the separation of the amlodipine, valsartan and hydrochlorothiazide were Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm) column and a mobile phase consisting of acetonitrile-0.1 M KH_2PO_4 pH 3.0-water (75:6:19, % v/v/v). Column temperature was maintained at 40 ^oC and the flow rate was 1ml/min. Detection was at 229 nm and injection of 20 µl.

The optimum retention time and resolution of amlodipine, valsartan and hydrochlorothiazide was determined using isocratic reversed phase HPLC method, by use of one organic modifier and buffer combination with all components eluting within 6 min. The column chosen was C-18 (250 mm × 4.6 mm i.d. 5 µm) because these types of columns are the ones routinely used in liquid chromatography analysis of pharmaceuticals. The order of elution of the component was hydrochlorothiazide, valsartan and amlodipine respectively. Detection was done at 229 nm. The study focused on optimization of the conditions for simple, rapid and cost effective analysis including selection of routinely used columns and mobile phases to obtain satisfactory results.

In the reported methods [33, 34] the column used was C-18 (150 mm × 4.6 mm i.d. 5 µm) which is not the routine practice. The elution time was long, the last component to elute having a retention time of 8.8 min and 10 min, respectively. The order of elution of the component was hydrochlorothiazide, amlodipine and valsartan, respectively [33] and amlodipine, valsartan and hydrochlorothiazide, respectively [34]. Detection was done at 239 nm and 238 nm, respectively.

This method developed for the analysis of multiple components in antihypertensive drugs exhibited good sensitivity, precision and linearity to allow for its use in the assay of these ingredients in commercial samples. By this simultaneous method, three compounds of interest

can be analyzed in a single HPLC run under isocratic conditions, the method greatly reducing overall analysis time from analyzing single compounds individually.

The development of this HPLC method aimed at using readily available and cheap reagents designed to make the procedure more routinely applicable and cost effective. Another factor aimed at making the method more readily applicable in a wide range of settings is its use of silica based reversed-phase C-18 column that is most commonly employed in the analysis of majority of drug compounds using HPLC techniques.

Indicative procedures on the method's robustness were tested by investigating the impact of varying key chromatographic factors including pH, acetonitrile concentration and column temperature on the variation of component peak areas and retention times. More comprehensive studies aimed at accessing the impact of simultaneous variation of these as well as other LC factors in the form of surface response plots would be a more accurate way of assessing the method's robustness.

6.2 Conclusion

A fast simple, reliable, precise and robust isocratic reverse phase HPLC method with UV detection was developed for the simultaneous determination of amlodipine, valsartan and hydrochlorothiazide. The optimized conditions for the separation of the 3 analytes amlodipine, valsartan and hydrochlorothiazide were: Hypersil C-18 (250 mm × 4.6 mm i.d. 5 µm) column, mobile phase: acetonitrile-0.1 M KH_2PO_4 pH 3.0-water, (75:6:19, % v/v/v). Column temperature maintained at 40 ^oC; Flow rate 1ml/min: Detection at 229 nm and injection of 20 µl, sample dissolved in mobile phase.

The method was validated for precision, specificity, accuracy and linearity. The precision of the method was shown through adequate repeatability or intraday precision (CV $\leq$ 2) and intraday

precision (CV ≤ 2). The method also demonstrated adequate linearity over the rage of 25-150%. The limit of detection for hydrochlorothiazide, valsartan and amlodipine were 10.72, 21.20 and 14.45 ng, while the limits of quantification were 35.76, 71.23 and 48.16 ng, respectively. The method also showed adequate robustness to small variations in mobile phase pH, column temperature and acetonitrile concentrations. The % recoveries of each added working standard for all compounds were within the specified guidelines (ICH) of 98-103% which showed that the method was accurate.

The run time was very short (6 min) which can allow rapid quantification of many samples in routine and quality control analysis of tablets. The developed method is rapid, selective, requires simple sample preparation procedures and simple mobile phase combinations. The method represents a good procedure for determination of hydrochlorothiazide, valsartan and amlodipine in bulk pharmaceuticals and dosage forms.

The method was applied in the assay of 6 commercial products containing all the three, combination of any two or any one of the three API obtained from randomly selected retail pharmacies located within the Thika town in the Kenyan market. Three batches of each product were analyzed. The most noticeable feature was the high content in most of samples of the amlodipine component whose assay value was found to be more than 110 % with content as high as 129.6 % thus failing to comply in seven batches out of nine with the assay limits defined in the B.P. and U.S.P. Hydrochlorothiazide in eight cases out of nine had low content than the specified in BP and USP. Valsartan complied in four batches from the twelve batches analyzed.

6.3 Recommendations

Collaborative studies between laboratories need to be carried out to access the ruggedness and reproducibility in order to improve the applicability of the method, making it routine and more versatile. These could establish the method's ruggedness across varying environments. Ruggedness is a pre-requisite for the successful transfer of any analytical technique to allow for widespread application of the method with a predictable degree of reliability and accuracy.

Stability indicating methods may be required for the quantitative determination of the purity of the drug substance and drug products in bulk samples and pharmaceutical dosage forms in the presence of its impurities and degradation products

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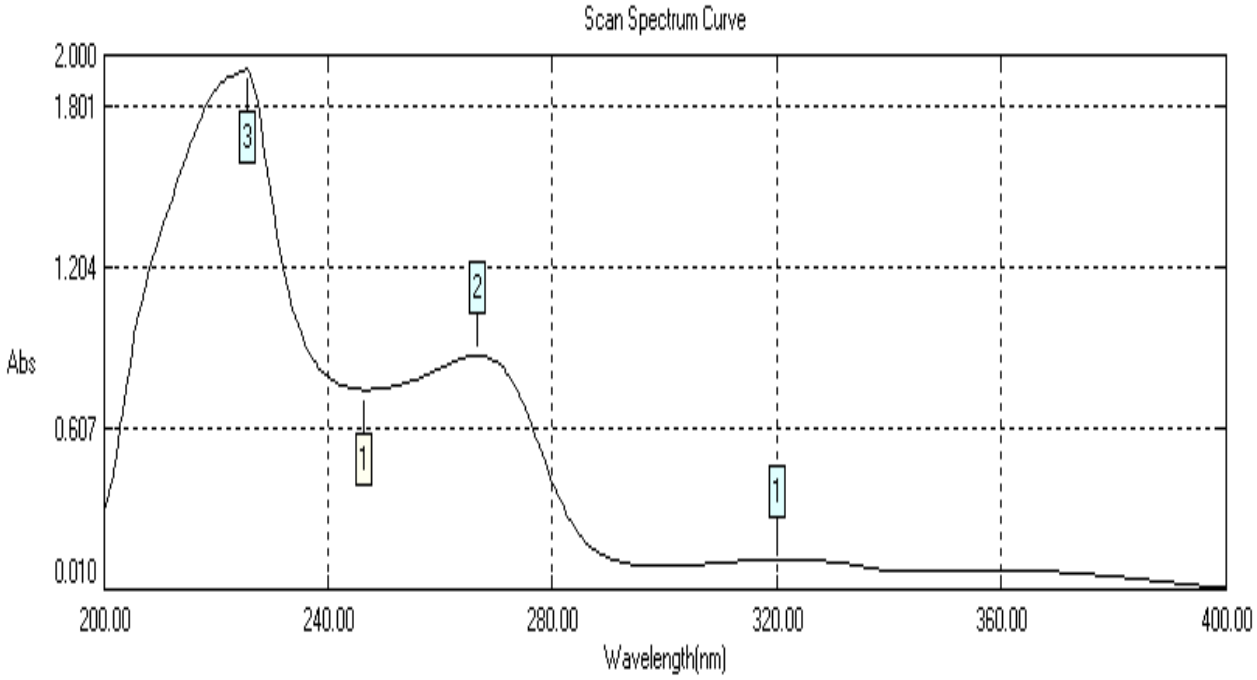
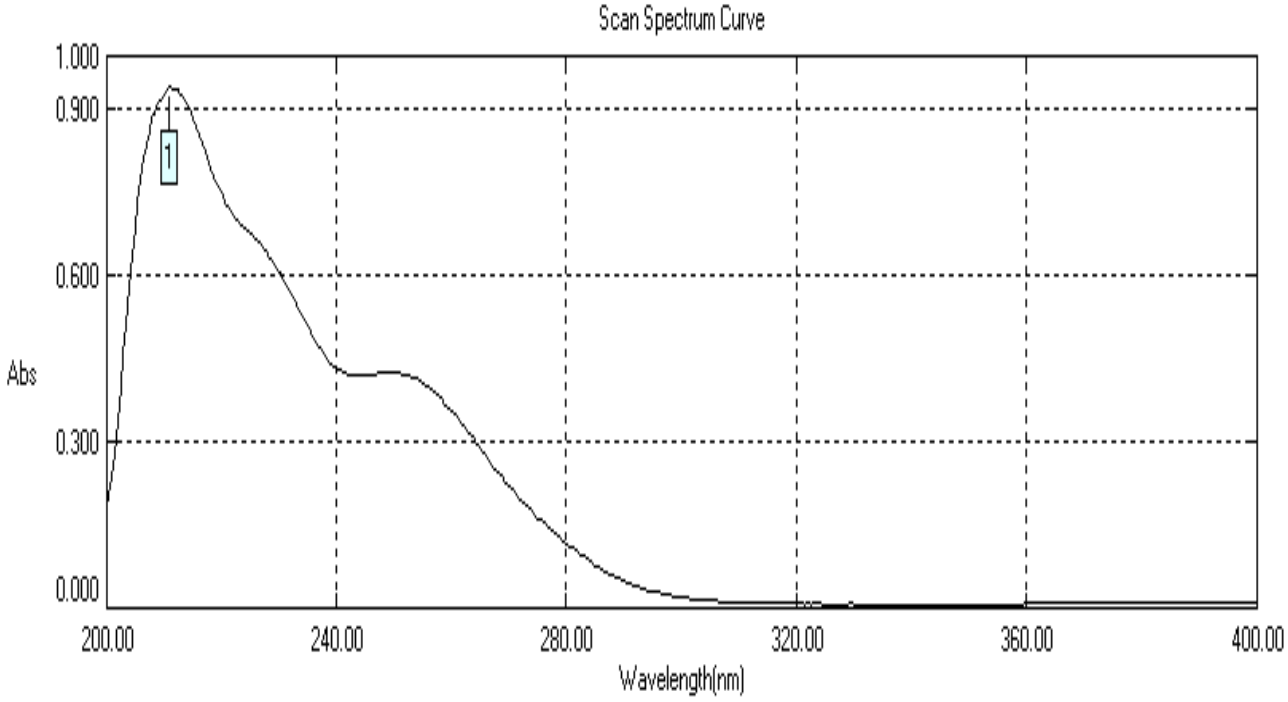
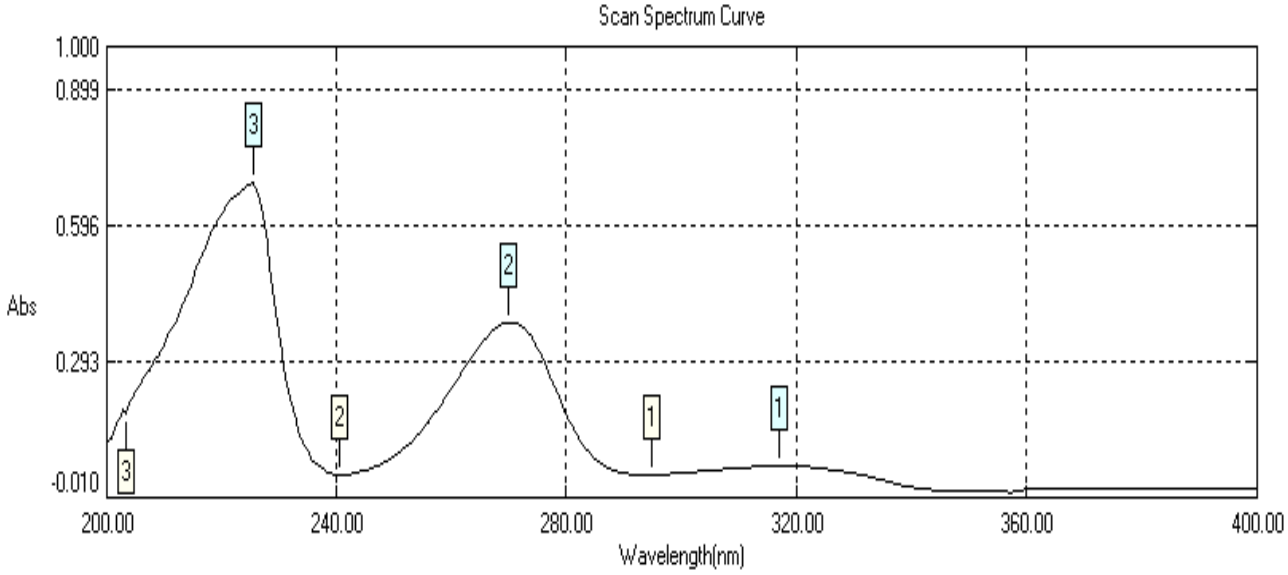
APPENDICES



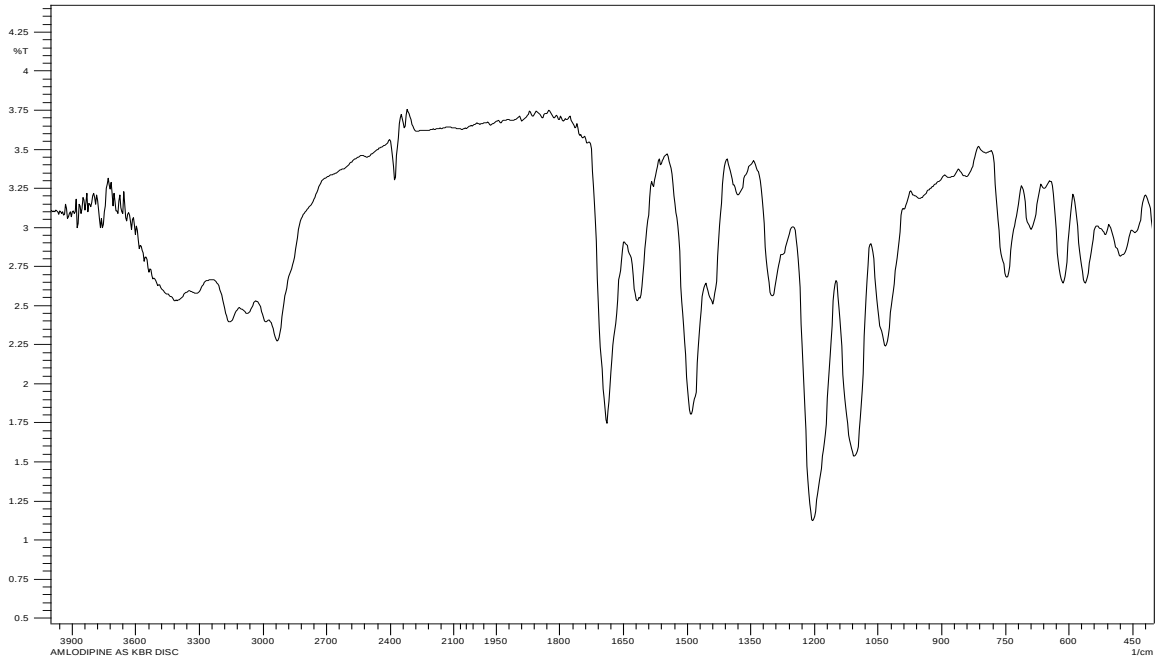
Appendix 1: UV spectrum of Amlodipine



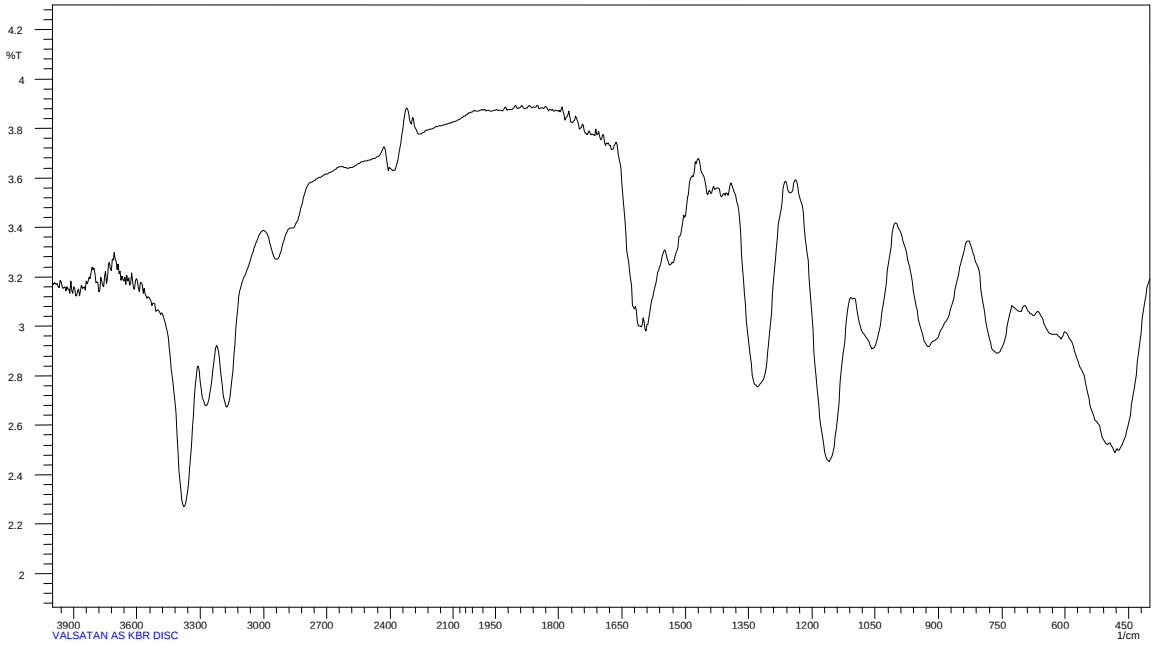
Appendix 2: UV spectrum of Valsartan



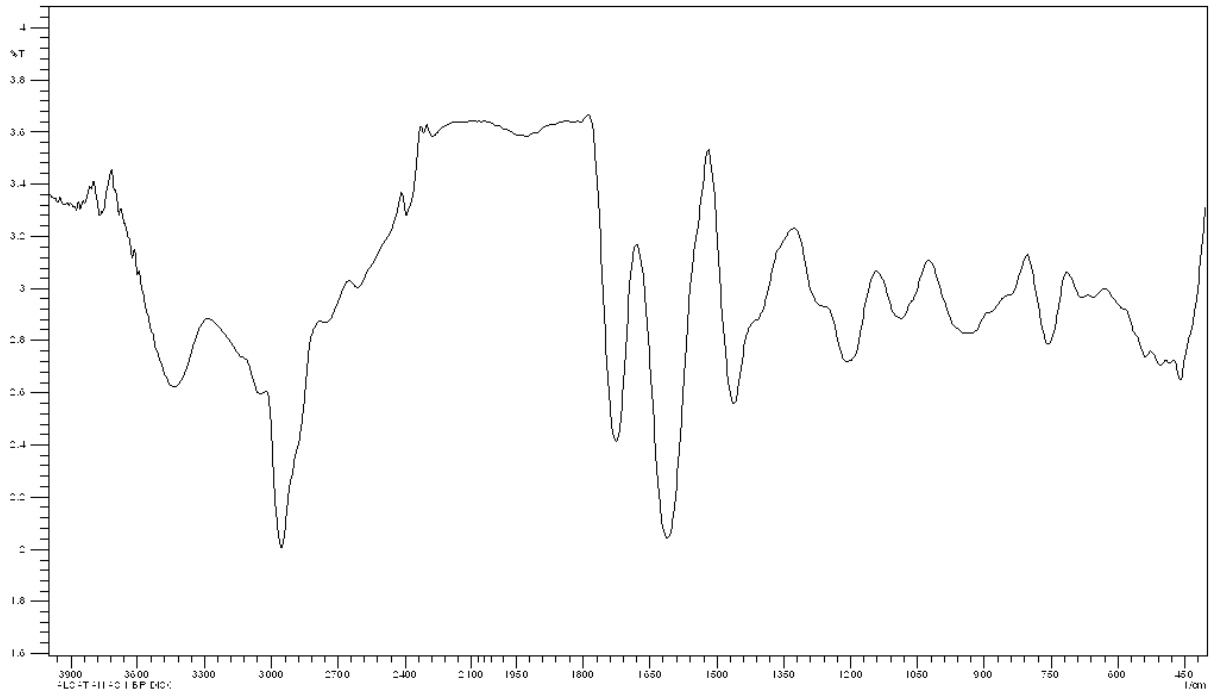
Appendix 3: UV spectrum of hydrochlorothiazide



Appendix 4: FTIR spectrum of amlodipine (KBr disk)



Appendix 5: FTIR spectrum of valsartan (KBr disk)



Appendix 6: FTIR spectrum of Hydrochlorothiazide (KBr disk)

Appendix 7: Details of robustness of the method for the hydrochlorothiazide, valsartan and amlodipine

Temperature ˚C	INJECTION	HCTZ		VAL		AMLO	
		PEAK AREAS	NPA	PEAK AREAS	NPA	PEAK AREAS	NPA
35	1	117972.9	118326.819	545575.8	546121.376	53702.8	53756.503
	2	117630.1	117982.99	540186.7	540726.887	53023.7	53076.724
	3	118439.6	118794.919	545509.2	546054.709	53058.1	53111.158
40	1	117002.1	117353.106	545509.2	546054.709	53058.1	53111.158
	2	117211.1	117562.733	545503.3	546048.803	53823.2	53877.023
	3	117166.1	117517.598	545251.1	545796.351	53939.5	53993.44
45	1	117311.6	117663.535	541792.3	542334.092	53866.7	53920.567
	2	117096.1	117447.388	539127.1	539666.227	53492.3	53545.792
	3	117566.4	117919.099	540533.3	541073.833	55999.5	56055.5

Conc of acetonitrile (%)	INJECTION	HCTZ		VAL		AMLO	
		PEAK AREAS	NPA	PEAK AREAS	NPA	PEAK AREAS	NPA
70	1	117696.2	118049.289	542027.3	542569.327	55230.2	55285.43
	2	117394.2	117746.383	551883.5	552435.384	55520.3	55575.82
	3	123210.1	123579.73	547854.3	548402.154	53598.8	53652.399
75	1	117369.1	117721.207	543310.5	543853.811	56667.8	56724.468
	2	118639.6	118995.519	542632.7	543175.333	54913.7	54968.614
	3	117371.1	117723.213	542645.9	543188.546	54900.6	54955.501
80	1	117883.5	118237.151	542326.7	542869.027	55394.3	55449.694
	2	117351.1	117703.153	545385.1	545930.485	55021.4	55076.421
	3	118965.3	119322.196	548964.8	549513.765	55575.4	55630.975

pH	INJECTION	HCTZ		VAL		AMLO	
		PEAK AREAS	NPA	PEAK AREAS	NPA	PEAK AREAS	NPA
2.75	1	122341.5	122708.525	552151.9	552704.052	56123.1	56179.223
	2	122843.9	123212.432	552976.4	553529.376	55470.2	55525.67
	3	122951.7	123320.555	556954.1	557511.054	56477.4	56533.877
3	1	122369.1	122736.207	543310.5	543853.811	56667.8	56724.468
	2	122639.6	123007.519	542632.7	543175.333	54913.7	54968.614
	3	121371.1	121735.213	542645.9	543188.546	54900.6	54955.501
3.25	1	122589.3	122957.068	558199.8	558758	54053.5	54107.554
	2	122455.8	122823.167	555071.9	555626.972	54979.7	55034.68
	3	122635.1	123003.005	555574.6	556130.175	54983.1	55038.083

NPA- Normalized peak areas

Appendix 8: Glossary

Antihypertensive:	A drug that reduces high blood pressure
Compliance:	Degree to which a patient correctly follows medical advice
Fixed Dose Combination:	Two or more active drugs in a single dosage form
Gradient:	Rate of increase or decrease of a variable value
Hemodynamic:	Study of the forces involved in blood circulation
Isocratic:	Mobile phase remains constant throughout the procedure
Morbidity:	Rate of incidence of a disease
Poly Pharmacy:	Use of multiple unnecessary medications
Precision:	Ability of a measurement to be consistently reproduced
Prevalence:	Total number of cases of a disease in a given population
Regimen:	Prescribed course of medical treatment, diet or exercise
Surveillance:	The act of observing a condition
Symphathoplegic:	Compounds that decrease sympathetic nervous system
Synergistic:	Increased activity of a drug on addition of other drugs
Tailing:	Chromatographic peak shape distortion